

**A ROLE FOR PFKFB3/IPFK2 IN OVERNUTRITION-ASSOCIATED ADIPOSE
TISSUE AND INTESTINE INFLAMMATORY RESPONSES AND INSULIN
RESISTANCE**

A Dissertation

by

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ABSTRACT

Overnutrition causes many metabolic diseases including type 2 diabetes. PFKFB3/iPFK2 is a master regulator of adipocyte and intestinal nutrient metabolism. Using PFKFB3/iPFK2^{+/-} mice and adipocyte-specific PFKFB3 over-expression mice, the present study investigated the role of PFKFB3/iPFK2 in regulating diet-induced adiposity, inflammation in adipose tissue and intestine, and systemic insulin resistance.

On a high-fat diet (HFD), PFKFB3^{+/-} mice gained much less body weight than did wild-type littermates. However, HFD-induced systemic insulin resistance in PFKFB3^{+/-} mice was more severe than in wild-type littermates. In contrast, adipocyte-specific PFKFB3 over-expression increased adiposity but suppressed overnutrition induced adipose tissue inflammatory response and improved insulin sensitivity. In addition to adipose tissue, PFKFB3/iPFK2 also played a role in intestine events. Compared to wild-type littermates, PFKFB3^{+/-} mice displayed a significant increase in the expression of intestinal inflammatory markers on a HFD.

In conclusion, PFKFB3 protects against overnutrition-induced adipose tissue and intestine inflammatory response and systemic insulin resistance in an adiposity-independent manner. Selective PFKFB3 activation may be viable for treating and/or preventing insulin resistance and type 2 diabetes.

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NOMENCLATURE

6PFK1	6-phosphofructo-1-kinase
AMPK	5' Adenosine Monophosphate-activated Protein Kinase
AP-1	Activator Protein 1
CPT1	Carnitine Palmitoyltransferase I
CRP	C-reactive Protein
Ctrl	Control
F2,6BP	Fructose 2,6-bisphosphate
FABPs	Fatty Acid Binding Proteins
FFAs	Free Fatty Acids
FAS	Fatty Acyl-CoA Synthase
GLUT4	Glucose Transporter 4
GTT	Glucose Tolerance Test
HFD	High Fat Diet
IKK	I κ B Kinase
IL-1ra	Interleukin-1 receptor antagonist
IL-6	Interleukin-6
IL-10	Interleukin-10
INS	Insulin
iPFK2	Inducible 6-phosphofructo-2-kinase
IR	Insulin Resistance
ITT	Insulin Tolerance Test

JNK	c-Jun N-terminal kinase
LXR	Liver X Receptor
MCP-1	Monocyte Chemotactic Protein-1
Nac	N-acetyl-L-cysteine
NF κ B	Nuclear Factor κ B
PGC1	PPAR Gamma Co-activator 1
PI3K	Phosphatidylinositol-3 Kinase
PPAR	Peroxisome Proliferator-activated Receptor
PPAR α	Peroxisome Proliferator-activated Receptor Alpha
PPAR γ	Peroxisome Proliferator-activated Receptor Gamma
PPREs	PPAR γ Response Elements
ROS	Reactive Oxygen Species
RT-PCR	Reverse Transcription Polymerase Chain Reaction
RXR	Retinoid X Receptor
T2DM	Type 2 Diabetes Mellitus
TG	Triglycerides
TNF α	Tumor Necrosis Factor α
TZDs	Thiazolidinediones
WAT	White Adipose Tissue

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CHAPTER I

INTRODUCTION

It has been shown that adipose tissue plays an essential role in the regulation of systemic metabolic homeostasis. In adipocytes, PFKFB3 is the gene that encodes for the inducible 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (iPFK2), which is a regulatory enzyme of glycolysis. In mouse models of diabetes and/or insulin resistance, the expression of adipose PFKFB3 is decreased, which is accompanied by hyperglycemia and insulin resistance. Upon the treatment of anti-diabetic agents, i.e., thiazolidinediones (TZDs), the expression of PFKFB3 in adipocytes is induced, which may be required, at least in part, for the effect of TZDs on restoring euglycemia and insulin sensitivity in mice. These data pointed to a pivotal role of adipose PFKFB3 in the regulation of systemic glucose homeostasis and insulin sensitivity. Recently, it was found that PFKFB3/iPFK2 is highly expressed in the intestine. Additionally, much evidence demonstrates that the intestine is involved in the regulation of glucose hemostasis and inflammatory responses. However, the mechanisms underlying these effects are unknown. Therefore, the project goal is to determine the role of PFKFB3/iPFK2 in overnutrition related adipose tissue and intestine inflammatory responses and insulin resistance. The central hypothesis of this project is that PFKFB3/iPFK2 coordinates metabolic and inflammatory responses in adipose tissue and intestine to modulate the generation of metabolism-regulator factors, and thereby brings

about inter-tissue effects to regulate systemic metabolic hemostasis. The significance of this work is the identification of the mechanisms underlying the pathogenesis of obesity and diabetes so that novel therapeutic targets can be found to treat obesity and diabetes. The general approach used to test the central hypothesis includes measurement of changes in biomarkers of metabolism and inflammation using loss-of-function of PFKFB3/iPFK2 and/or gain-of-function of PFKFB3/iPFK2 in mice. The approach is innovative because it is unique to use PFKFB3/iPFK2-knockout mice model and PFKFB3/iPFK2-adipocyte-specific overexpressing mice model. The predicted outcome is that PFKFB3/iPFK2, although contributing to adiposity, protects against diet-induced adipose tissue and intestine inflammatory responses, and improves insulin sensitivity.

CHAPTER II

LITERATURE REVIEW

METABOLIC SYNDROME, OBESITY, AND TYPE 2 DIABETES

Leading causes of death altered in US compared to a hundred years ago. The top 10 causes of death shifted from infection-related diseases such as pneumonia, influenza, tuberculosis and enteritis to nutrition-related diseases such as heart disease, cancer, stroke, diabetes [1]. Diabetes became the seventh leading cause of death, as shown in Figure 1. In the US, type 2 diabetes accounts for 95% of all diabetes cases, and is associated with obesity. Type 2 Diabetes Mellitus (T2DM) is recognized to be a potential killer, because it can be latent for many years and neglected by people. Millions of people may have undiagnosed diabetes, and millions of people have pre-diabetes. In recent decades, more and more adolescences and children are diagnosed with type 2 diabetes [2].

Metabolic syndrome is a condition with insulin resistance and other metabolic or vascular disorders. The characteristic features of metabolic syndrome, as shown in Figure 2, include central obesity, insulin resistance, hypertension, dyslipidemia, atherosclerosis, and certain forms of cancers [3-5]. Overnutrition or obesity has been considered one of causing factors of metabolic syndrome. Weight loss due to a very-low-calorie-diet can improve several aspects of metabolic syndrome such as reduction of

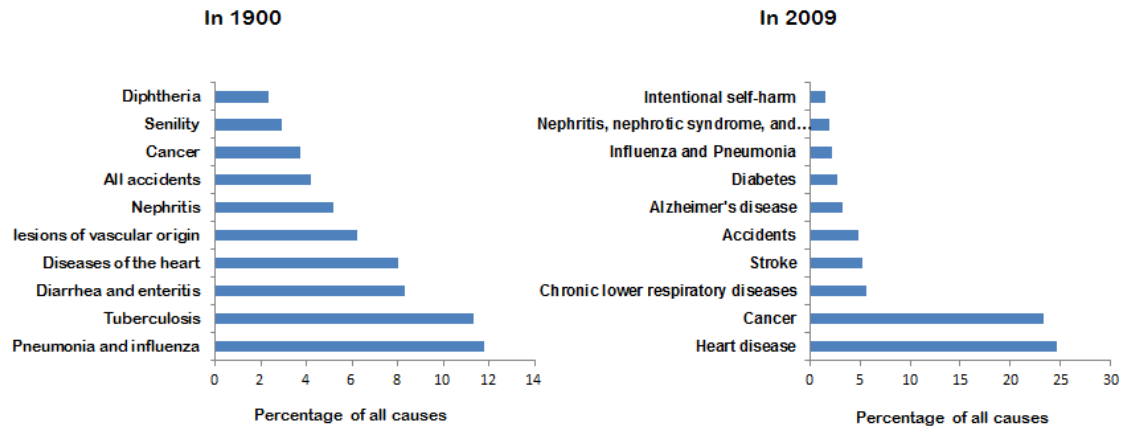


Figure 1. Leading Causes of Death in the US, 1900 vs. 2009. [1]

blood pressure, glucose, triglycerides and cholesterol [6]. Insulin is an important hormone that facilitates the uptake and metabolism of glucose in muscle and adipose tissue. Insulin resistance is a physiological condition that normal insulin function is impaired and becomes less effective to lower blood glucose. Central obesity, physical inactivity and genetic factors are main reasons leading to the development of insulin resistance. Insulin resistance has been considered an important factor developing metabolic syndrome and connecting many of the features of metabolic syndrome [7]. However, recent studies suggest that inflammation is associated with the pathogenesis of metabolic syndrome [8], as shown in Figure 3. Nutrients overload, physical inactivity, and/or genetic factors result in obesity, which is characterized by a low-grade chronic inflammation. The later is displayed by increased production of inflammatory cytokines such as Tumor Necrosis Factor Alpha (TNF α) and Interleukin 6 (IL-6) via enhancing the

generation of Reactive Oxygen Species (ROS) and stimulating Nuclear Factor- κ B (NF- κ B) signal pathway[9]. Inflammation has been found to cause insulin resistance, atherosclerosis, and certain forms of cancers. Insulin resistance also exacerbates inflammatory responses, which forms a vicious cycle and leads to metabolic syndrome [8-10].

INFLAMMATION: A CAUSAL FACTOR OF OBESITY-RELATED INSULIN RESISTANCE AND DIABETES

Increasing plasma levels of inflammatory markers such as TNF α , IL-6, leptin, and C-Reactive Protein (CRP) in overweight and obese animals and subjects indicate that obesity can be considered as a low-grade systemic inflammatory disease [11-14]. White Adipose Tissue (WAT) of obese human and animals is characterized by elevated production and secretion of inflammatory factors including TNF α and IL-6 [15, 16]. Macrophage infiltration in adipose tissue due to adipose tissue dysfunction is suggested to produce prominent source of pro-inflammatory cytokines in adipose tissue [17, 18]. Inflammatory factors produced and secreted from both adipocytes and macrophages have effects not only on adipose physiological functions, but also on other organs such as liver and muscle, and contribute to the pathogenesis of insulin resistance [19, 20].

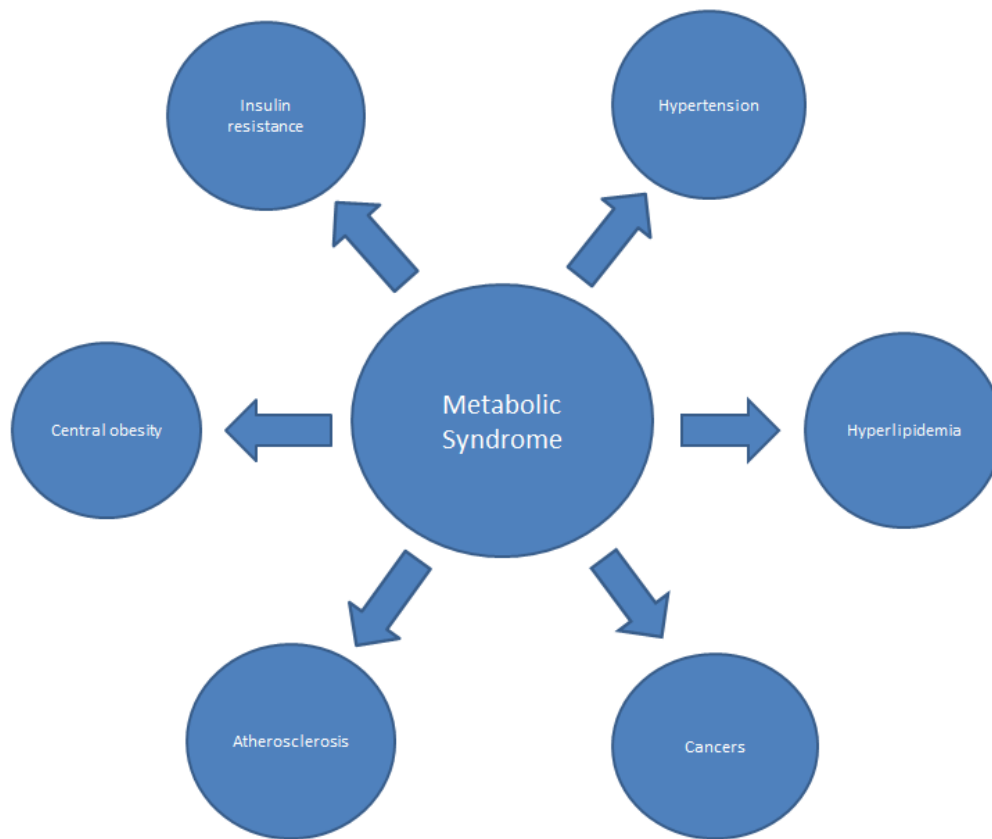


Figure 2. Characteristic Features of Metabolic Syndrome.

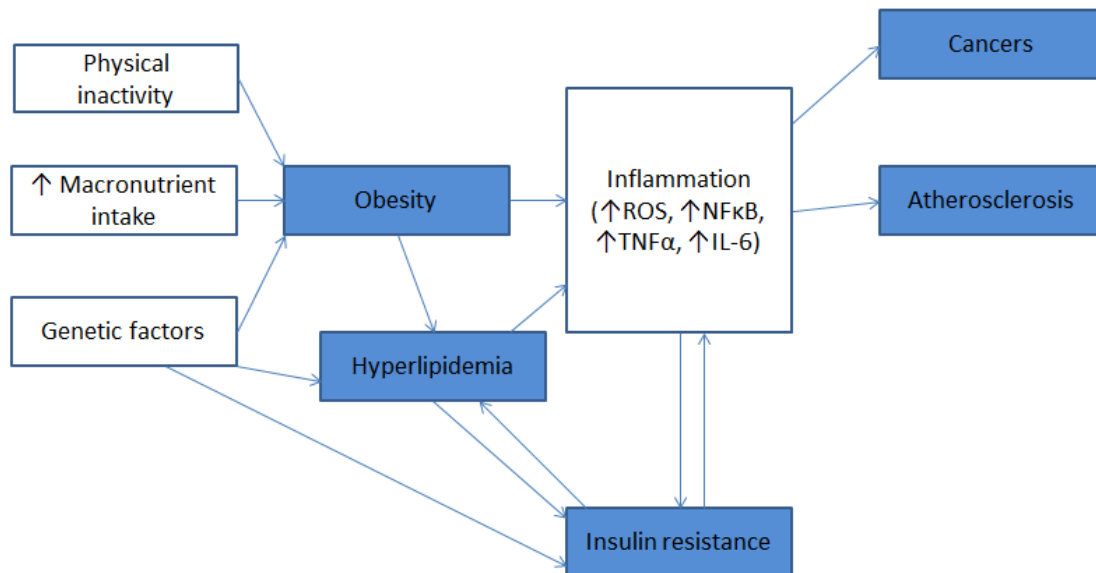


Figure 3. Pathogenesis of Metabolic Syndrome.[8]

Many studies suggest that anti-inflammatory approaches are effective in terms of reversing systemic insulin resistance and improving systemic metabolic homeostasis [21]. Exercise, a way to improve insulin sensitivity, can enhance anti-inflammatory effects via increasing levels of anti-inflammatory cytokines and reducing levels of pro-inflammatory cytokines. For example, IL-6 produced by muscle during exercise stimulates the production of anti-inflammatory factors such as Interleukin-1 receptor antagonist (IL-1ra) and Interleukin-10 (IL-10), which can inhibit the production of TNF α [22, 23]. In addition, exercise suppresses the production of inflammatory monocytes such as CD14⁺ and CD16⁺ cells [24]. Insulin-sensitizing drugs —

thiazolidinediones (TZDs) such as troglitazone, rosiglitazone and pioglitazone, have anti-inflammatory effects via activation of PPAR γ and suppression of inflammatory cytokines [25-27]. Metformin, an anti-diabetic drug, exerts anti-inflammatory effects by inhibition of NF κ B, TNF α and IL-6 production via blockade of Phosphatidylinositol-3 Kinase (PI3K)-Akt pathway and PI3K-dependent 5' Adenosine Monophosphate-activated Protein Kinase (AMPK) phosphorylation [28-30]. Some nutrients have impact on insulin action. Omega-3 fatty acids such as Docosahexaenoic Acid (DHA) and Eicosapentaenoic Acid (EPA) exhibit anti-inflammatory effects and enhance insulin sensitivity via stimulation of G Protein-coupled Receptor 120 (GPR120) [31, 32]. Anti-oxidant vitamins (Vitamin E and C) exhibit anti-inflammatory effects and improve insulin sensitivity by inhibition of oxidative stress [33]. Dietary flavonoids, which are ubiquitous in plants such as nuts, seeds, fruits and vegetables, have antioxidant and anti-inflammatory activities which provide benefits on chronic inflammatory diseases [34].

Elevated serum levels of inflammatory markers such as CRP, IL-6, IL-1, and white cell count are associated with T2DM [35]. Some T2DM prevention programs including metformin therapy and lifestyle intervention significantly reduce inflammatory markers [36]. Proinflammatory cytokines derived from adipose tissue such as IL-6 and TNF α have been shown to play a key role in causing insulin resistance [19, 37]. Therefore, it is evident that there is a link between inflammation and pathogenesis of insulin resistance and T2DM.

The mechanisms underlying the link between the inflammatory response and the pathogenesis of insulin resistance is involved several molecule pathways, as shown in

Figure 4. In normal insulin action, insulin binds to insulin receptor and triggers PI3K-Akt pathway via insulin receptor substrate (IRS). Akt is phosphorylated and activated. Active Akt mediates many downstream signals, and targets a series of substrates that govern cell metabolism and proliferation [38-40]. For example, Akt phosphorylation mediates translocation of Glucose Transporter 4 (GLUT4) from cytosol to membrane. GLUT4 is an important transporter for glucose uptake in adipocytes and muscle cells [39]. Blockade of IRS function impairs insulin signal pathway. Therefore, IRS is a crucial signal molecule for insulin action. Inflammatory cytokine, ROS and lipid products activate c-Jun N-terminal Kinases (JNK) and result in serine phosphorylation of IRS-1 and 2, thus inhibiting insulin signaling. Activation of I κ B Kinase (IKK) phosphorylates I κ B α which releases NF- κ B to the nucleus. NF- κ B triggers a series of transcriptional events to produce inflammatory cytokines. Therefore, many more inflammatory cytokines activate JNK and IKK, exacerbating insulin resistance. JNK also controls the expression of inflammatory genes through transcription factor Activator Protein 1(AP-1). Peroxisome proliferator-activated receptor (PPAR) and Liver X Receptor (LXR) families transcript genes of metabolism inhibit inflammatory activity. Fatty Acid Binding Proteins (FABPs) block PPAR- and LXR-mediated transcriptional events through regulating lipids activities [9, 41, 42].

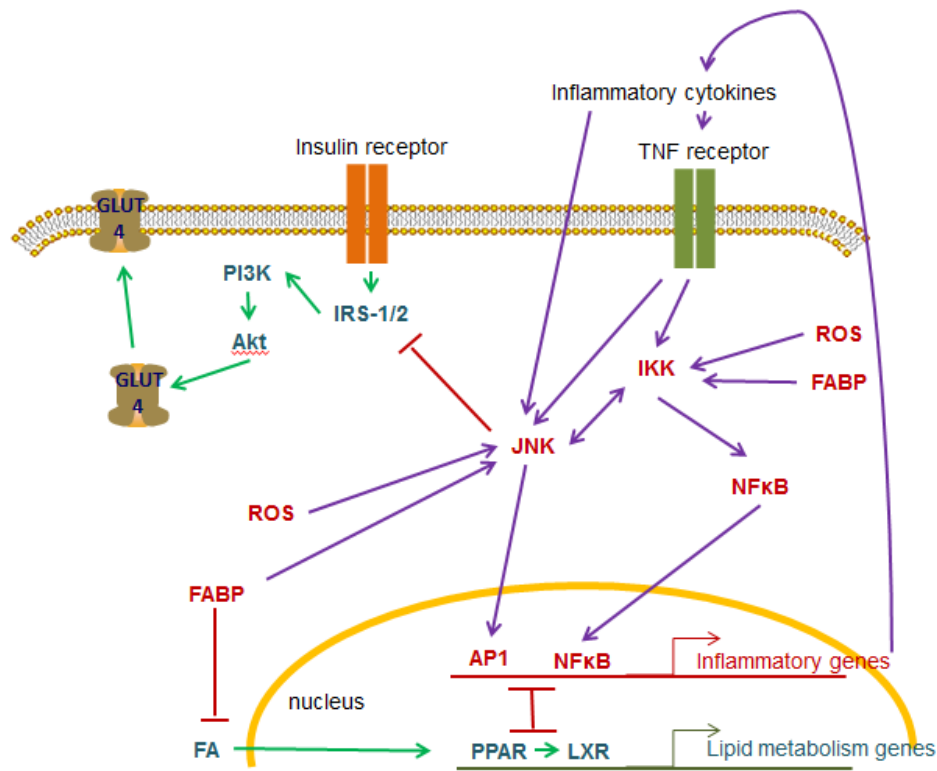


Figure 4. Molecular Pathways Linking Inflammatory Response and Insulin Action.
[9, 41]

ADIPOSE TISSUE FUNCTION AND INFLAMMATION

White adipose tissue is an important metabolic organ, which stores energy when energy balance is positive and releases energy when energy balance is negative [43, 44]. In the process of storing energy, Free Fatty Acids (FFAs) are generated as the products of Triglyceride (TG) hydrolysis during delivery of both dietary fats in the form of chylomicrons and endogenous fats in the form of very low density lipoproteins in

response to feeding. FFAs, along with glycerol as additional products of triglyceride hydrolysis, are then transported into adipocytes via a transport complex. Inside adipocytes, free fatty acids are re-esterified and the resultant triglycerides are stored in lipid droplets.

Adipose tissue is also an endocrine organ, whose endocrine functions tightly control systemic metabolic homeostasis. In fact, the balance between the release of pro-hyperglycemic factors such as FFAs and resistin and anti-hyperglycemic factors such as adiponectin, as well as the production of proinflammatory cytokines such as tumor necrosis factor alpha (TNF α) and interleukin 6 (IL-6) from both adipocytes and adipose tissue macrophages and other immune cells, determine insulin sensitivity and glucose metabolism locally in adipose tissue and distally in liver and skeletal muscle [45-48]. Of significance, the endocrine function of adipose tissue is tied closely with adipocyte glucose and lipid metabolism.

Adipose tissue has an important relevance to metabolic disease. Much evidence suggests that adipose tissue dysfunction is a major causing factor of insulin resistance [49-51]. White adipose tissue dysfunction is prominently characterized by decreased capability of fat storage which results in excessive energies (glucose and fatty acids) likely because nutrients cannot be converted to TG for storage in fat cells, rather stay in circulation. Organs are exposed in high glucose and fatty acids, leading to decrease insulin signal pathway or insulin resistance. Meanwhile, accumulation of TG in other organs destroys functions of organs, inducing various metabolic diseases. For example,

accumulation of fat in liver causes fatty liver which can result in Steatohepatitis and even cirrhosis [52, 53]

Many studies showed that inflammation is one of characters in metabolic dysregulation of white adipose tissue [16, 54-58]. Overnutrition causes excessive accumulation of TG in fat cells, which enlarges their sizes. Once the fat cells continuously enlarge, functions of fat cells are impaired. Fat cells release many cytokines such as TNF α and Monocyte Chemotactic Protein-1 (MCP-1), which attracts macrophages and promotes macrophage infiltration in adipose tissue, as shown in Figure 5. Inside adipose tissue, macrophages release many more inflammatory factors that, in turn, impair insulin signaling in adipose tissue and promote insulin resistance. Because of insulin resistance in adipose tissue, fat cells cannot normally intake and metabolize glucose and store energy. This causes metabolic dysregulation of adipose tissue, which contributes to systemic metabolic dysregulation and insulin resistance [18, 59].

INTESTINAL FUNCTION AND INFLAMMATION

There are many studies focusing on metabolic disorders in liver, adipose tissue and skeletal muscle. However, little studies investigated the role of the intestine in overnutrition-related metabolic disorders. Intestine is the organ for digestion, absorption and assimilation of nutrients. Physiologically, nutrients absorbed by intestine, along with a number of nutritional and hormonal signals, are delivered to both central and peripheral tissues in response to feeding. This leads to appropriate regulation of nutrient metabolism in key metabolic tissues such as liver, adipose tissue, and skeletal muscle to

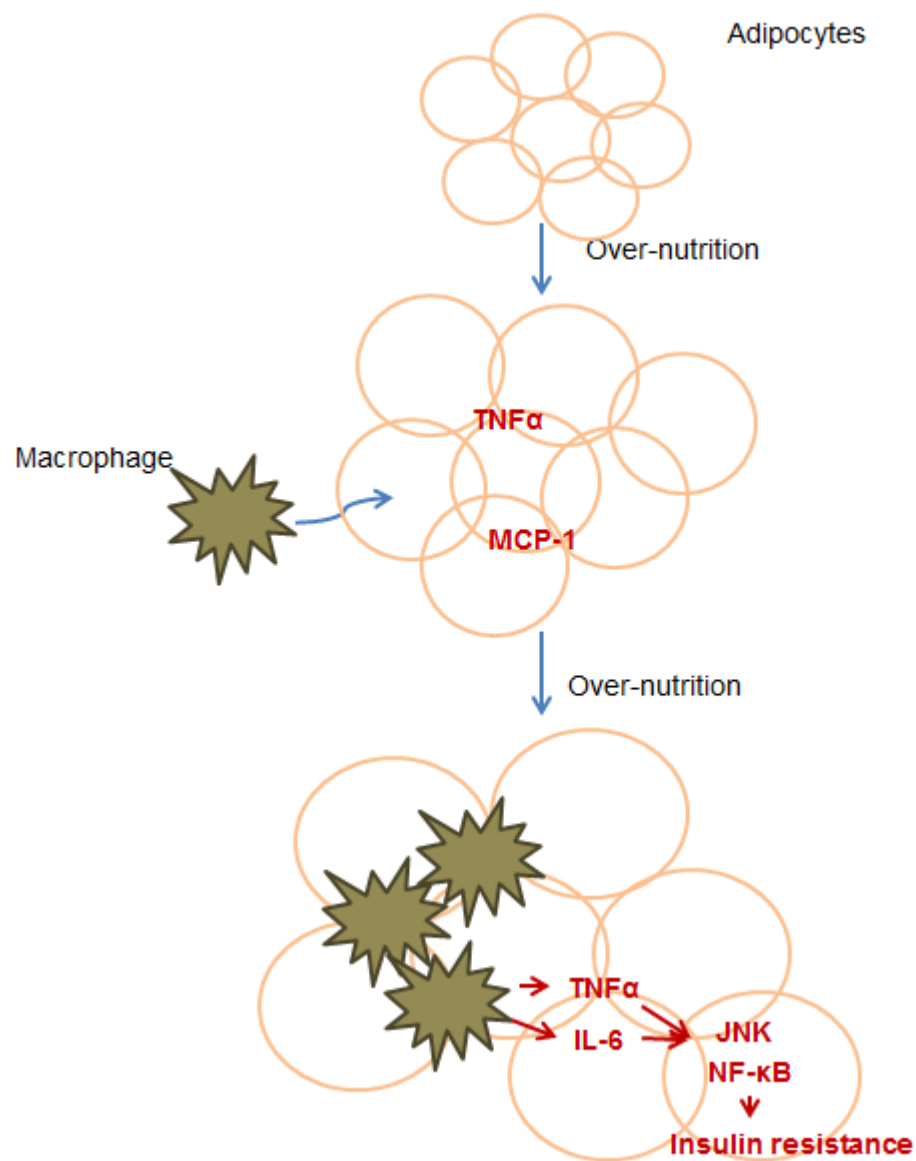


Figure 5. Adipose Tissue Inflammation and Macrophage Infiltration in Obesity.[16]

maintain systemic metabolic homeostasis and insulin sensitivity [60, 61]. For example, incretin hormones secreted by intestine facilitate glucose uptake by muscle and suppress the production of glucose in liver [62, 63]. The hormones secreted by the intestine can control not only the intestinal motility and satiety but also the glucose homeostasis [64]. In HFD induced obesity and insulin resistance mouse models, many genes related to lipid metabolism are altered in the small intestine, and are associated with insulin resistance [65]. Pathologically, nutrient overload disturbs glucose and lipid metabolism and triggers the inflammatory response in intestine. For example, feeding a High-Fat Diet (HFD) to mice activates NF- κ B activity in intestine cells including epithelial cells, immune cells, and endothelial cells of small intestine[66], which appears to contribute to HFD-induced insulin resistance and adiposity.

Intestine also hosts microbes, whose composition, when altered by overnutrition, contributes to increased intestine inflammatory response and the development of systemic insulin resistance [66-69]. Failure to maintain the balance causes impairment of intestinal homeostasis, even systemic homeostasis [70]. Under overnutrition, gut microbiota are modified and promote the secretion of proinflammatory cytokines that are associated with the development and progression of insulin resistance and metabolic disorder [66, 71]. Mechanisms of the control of inflammation by microbiota could be due to that changes in microbiota increase intestinal permeability [72, 73].

At this point, little is known about how glucose and lipid metabolism is orchestrated to regulate intestine inflammatory response. To understand the link between intestine nutrient metabolism and the inflammatory response in intestine is of particular

importance to a better understanding of the patho-physiology of overnutrition-associated insulin resistance and inflammatory intestine diseases.

PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR γ (PPAR γ)

Peroxisome proliferator-activated receptors (PPARs) are nuclear receptors. There are three PPAR isotypes are found: α , β , and γ [74]. PPAR α is highly expressed in the liver and brown adipose tissue. PPAR β is expressed most in kidney, heart and gut. PPAR γ is mainly expressed in adipose tissue [74, 75]. Many researches have shown that PPAR γ activated by TZDs effectively improves systemic insulin sensitivity and lowers plasma glucose levels in both human patients and rodent models of type 2 diabetes [76-82]. In nucleus, PPAR γ forms a heterodimer with the Retinoid X Receptor (RXR). When TZDs binds to PPAR γ , a structure change promotes the binding of the PPAR γ -RXR complex to PPAR γ Response Elements (PPREs) in target genes and changes of the transcription of these genes [83-85]. PPREs are found in many genes involved in glucose metabolism and lipid metabolism, such as GLUT4 transporter, glucokinase, fatty acids transporter protein, lipoprotein lipase, and Fatty Acyl-CoA Synthase (FAS) [85, 86].

Because TZDs are effective in liver- or muscle-specific PPAR γ -deficient mice [87, 88] but not in adipose tissue-deleted mice [89] and adipose-specific PPAR γ -deficient mice [90], adipose tissue has been considered as the primary target site for the anti-diabetic effect of PPAR γ activation [91, 92]. Further investigations have suggested two adipose tissue-based mechanisms to largely explain the anti-diabetic effects of PPAR γ activation [45, 81], as shown in Figure 6. In the first mechanism, PPAR γ

activation by TZDs appropriately alters the expression of adipocyte genes that are involved in lipogenesis and TG synthesis to increase the capacity of fat storage in adipose tissue [93-96]. This leads to reduction of the circulating levels of FFAs and thereby reversal of FFAs-induced insulin resistance by the effect on systemic glucose metabolism, including suppression of hepatic glucose output and increasing glucose uptake by muscle. In the second mechanism, PPAR γ activation by TZDs suppresses the adipose tissue inflammatory response [58, 97] and appropriately regulates adipokine expression [81, 98, 99]. This improves adipose tissue function, which in turn brings about the insulin-sensitizing effect [81]. However, the molecular link between the two mechanisms remains to be elucidated.

Upon activation of PPAR γ , a number of PPAR γ target genes are altered to promote fat storage in adipose tissue [100-103]. For example, TZDs stimulate the expression of Glycerol Kinase (GyK), which increases triglyceride synthesis in adipocytes by providing glycerol 3-phosphate as a key substrate [100]. Similarly, PPAR γ activation stimulates the expression of the cyto-solic isoform of Phosphoenolpyruvate Carboxykinase (PEPCK) in adipocytes [101, 102]. This increases glyceroneogenesis and provides another way to synthesize glycerol 3-phosphate and consequently triglycerides in adipocytes/adipose tissue [104, 105]. At this point, although the stimulatory effect of

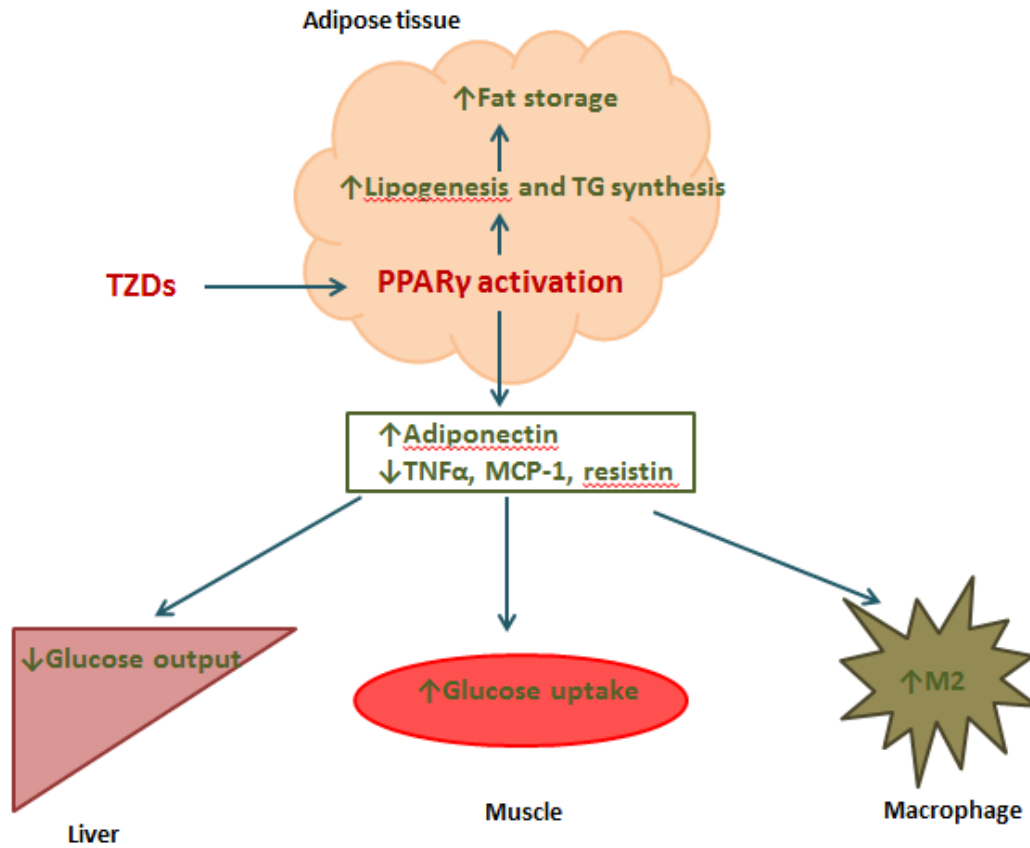


Figure 6. Mechanisms for Anti-diabetic Effects of PPAR γ Activation. [99]

PPAR γ activation on GyK and PEPCK expression is increasingly documented, the involvement of GyK and/or PEPCK in the antidiabetic effect of PPAR γ activation requires further exploration [106, 107]. Furthermore, there are no published data to address whether or not GyK and/or PEPCK are involved in the anti-inflammatory effect of PPAR γ activation. In response to TZDs, the expression of several proinflammatory

genes is decreased in the adipose tissue in both rodents and human patients with type 2 diabetes [95, 108]. Further, PPAR γ activation in both adipose tissue macrophages and adipocytes contributes to the suppression of the adipose tissue inflammatory response [97, 109]. To date, mediators that are involved in the effect of PPAR γ activation on adipocyte inflammatory response are largely unknown.

PFKFB3/iPFK2

PFKFB3 is the gene that encodes for the inducible 6-phosphofructo-2-kinase (iPFK2) that is highly expressed in adipose tissue [110]. PFKFB3/iPFK2 generates fructose-2,6-bisphosphate (F2,6P₂), which in turn activates 6-phosphofructo-1-kinase (6PFK1) to enhance glycolysis [111-113]. This effect is involved in adipocyte lipogenesis and triglyceride synthesis [110].

As shown in Figure 7, glucose uptake following insulin-stimulated GLUT4 translocation provides sufficient substrate that can be metabolized through glycolysis to produce glycerol-3-phosphate. Moreover, adipocyte glycolysis also generates pyruvate whose further metabolism in mitochondrial provides acetyl-CoA. The latter is used for generation free fatty acids through lipogenesis, and then triglycerides. In adipocytes, hexokinase catalyzes glucose phosphorylation. This step, however, is not a rate-limiting step. Instead, generation of F1,6P₂ from F6P is the rate-limiting step. F2,6P₂ activates 6PFK1 to enhance glycolysis in adipocytes. iPFK2, encoded by PFKFB3 [110] is the enzyme that generates F2,6P₂. Given this, glycolysis is a key to storing energy in

adipocytes. In support of this, iPFK2 is necessary for an increase in fat storage in white adipose tissue and in fat deposition in adipocytes.

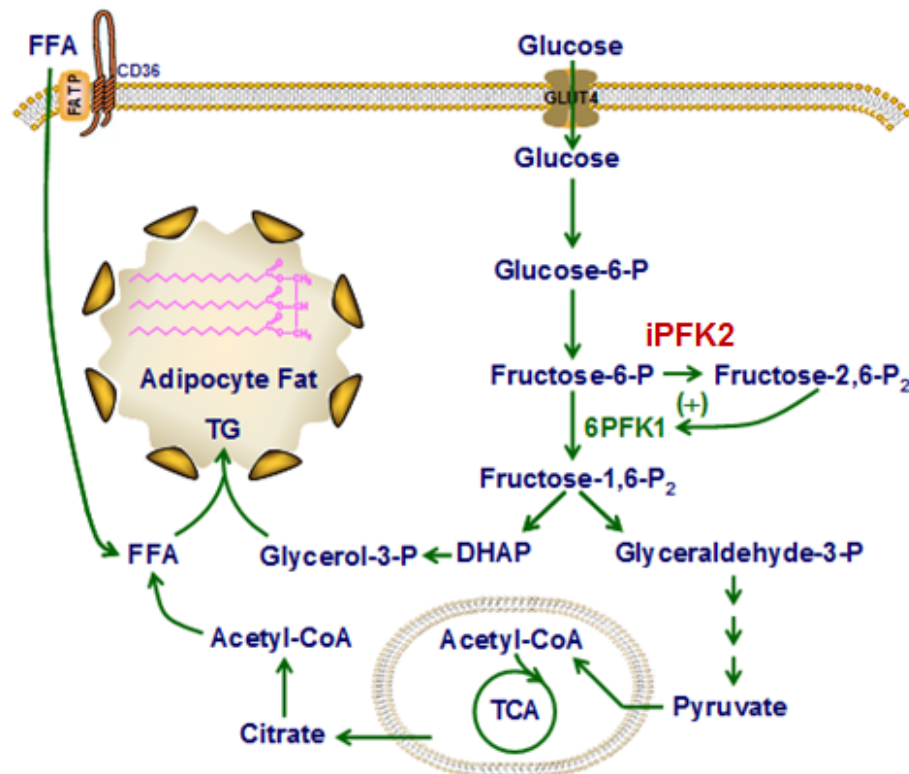


Figure 7. PFKFB3/iPFK2 Coordinates Adipocyte Glucose and Lipid Metabolism.

However, it is unknown whether the metabolic properties of PFKFB3/iPFK2 are related to the regulation of adipose tissue function, in particular adipose tissue inflammatory response. The present study provides evidence to support a novel role for

PFKFB3/iPFK2 in regulating diet-induced systemic insulin resistance and adipose tissue inflammatory response in a manner independent of adiposity.

SUMMARY

Diabetes became the seventh leading cause of death in the US. Additionally, 95% of diabetes is type 2 diabetes, which is mostly associated with obesity. Millions of people may have undiagnosed diabetes, and millions of people have pre-diabetes. Type 2 diabetes is characterized by insulin resistance. Nutrients overload, physical inactivity, and/or genetic factors result in obesity; this is characterized by a low-grade chronic inflammation. Inflammation contributes to insulin resistance. The later also exacerbates inflammatory response.

Mechanisms underlying the link between the inflammatory response and the pathogenesis of insulin resistance are associated with several molecule pathways. In normal insulin action, insulin binds to insulin receptor and triggers PI3K-Akt pathway via insulin receptor substrate (IRS). Inflammatory cytokines, ROS and lipid products activate c-Jun N-terminal Kinases (JNK) and result in serine phosphorylation of IRS-1 and 2 which inhibits insulin signaling. Activation of I κ B Kinase (IKK) phosphorylates I κ B α which releases NF- κ B to nucleus. NF- κ B triggers a series of transcriptional events to produce inflammatory cytokines. Therefore, many more inflammatory cytokines activate JNK and IKK, which exacerbate insulin resistance.

Peroxisome proliferator-activated receptor γ (PPAR γ) is nuclear receptors whose activation by TZDs effectively improves systemic insulin sensitivity and lowers plasma

glucose levels. In response to TZDs, the expression of several proinflammatory genes is decreased in the adipose tissue in both rodents and human patients with type 2 diabetes. Further, PPAR γ activation in both adipose tissue macrophages and adipocytes contributes to the suppression of the adipose tissue inflammatory response. However, mediators that are involved in the effect of PPAR γ activation on adipocyte inflammatory response are largely unknown.

PFKFB3 is the gene that encodes for the inducible 6-phosphofructo-2-kinase (iPFK2) that is highly expressed in adipose tissue. PFKFB3/iPFK2 enhances glycolysis which is involved in adipocyte lipogenesis and triglyceride synthesis. iPFK2 has been found to be necessary for an increase in fat storage in adipose tissue and in fat deposition in adipocytes. White adipose tissue is an important metabolic organ, as well as an endocrine organ. Adipose tissue dysfunction has been found to be the causing factor of inducing and developing insulin resistance. Inflammation is one of characters in metabolic dysregulation of white adipose tissue. PFKFB3 may play a role in adipose tissue function and inflammation. Recently, it was shown that PFKFB3 is also expressed in small intestine. Intestine is the organ for digestion, absorption and assimilation of nutrients. Nutrient overload disturbs glucose and lipid metabolism and triggers the inflammatory response in intestine. PFKFB3 regulates glucose and lipid metabolism, and appears to be associated with intestine inflammation.

CHAPTER III

THE ROLE OF PFKFB3/iPFK2 IN MOUSE ADIPOSE TISSUE METABOLISM, INFLAMMATORY RESPONSE, AND SYSTEMIC INSULIN RESISTANCE*

INTRODUCTION

Many studies demonstrated that overnutrition is associated with adiposity and systemic insulin resistance. For example, feeding an HFD to rats for 10 weeks causes a significant increase in visceral fat mass, which is accompanied by a decrease in systemic insulin sensitivity [114]. Similarly, after a feeding of HFD for 20 weeks, two different strains of wild-type mice gain a significant increase in fat mass, which is associated with systemic insulin resistance and glucose intolerance [115]. A recent study even indicates that feeding a HFD to mice for only 6 weeks is sufficient to induce adiposity and systemic insulin resistance [116]. Furthermore, as documented in mice lacking the myotonic dystrophy protein kinase, a larger augmentation in adiposity is accompanied

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by a greater increase in the severity of systemic insulin resistance [116]. Because of this, adiposity has been generally viewed as an important contributor of systemic insulin resistance [46, 116-119]. On the other hand, reducing adiposity has been considered as an effective way to reverse systemic insulin resistance [93, 114]. However, adiposity is not necessarily associated with systemic insulin resistance. This is particularly true in genetically modified mice and in mice treated with pharmacological agents [97, 120-123]. Following the investigation into the insight of altered systemic insulin sensitivity, it has been suggested that adipose tissue dysfunction is far more important than adiposity in terms of causing systemic insulin resistance [45, 81, 97, 124].

Mounting evidence points to a pivotal role for overnutrition-related inflammation in causing adipose tissue dysfunction, and thereby systemic insulin resistance. In mice fed an HFD, chronic low-grade inflammation in adipose tissue is evident and characterized by an increase in macrophage infiltration and proinflammatory cytokine production [125, 126]. This brings about adipose tissue dysfunction, demonstrated by an increase in the production pro-hyperglycemic factors such as free fatty acids and resistin and a decrease in the production of anti-hyperglycemic factors such as adiponectin [45-48]. These changes, along with increased production of proinflammatory cytokines such as tumor necrosis factor α (TNF α) and interleukin 6 (IL-6) from both adipocytes and adipose tissue macrophages, impair insulin signaling in insulin-sensitive tissues including the liver and skeletal muscle, leading to systemic insulin resistance [127-132]. In contrast, treatment with thiazolidinediones ameliorates adipose tissue inflammation,

which in turn contributes, at least in part, to the reversal of diet-induced adipose tissue dysfunction and systemic insulin resistance [97, 108]. For this reason, adipose tissue inflammation is of particular importance to the regulation of systemic insulin sensitivity.

PFKFB3 is the gene that encodes for the inducible 6-phosphofructo-2-kinase (iPFK2) that is highly expressed in adipose tissue [110]. PFKFB3/iPFK2 generates fructose-2,6-bisphosphate (F26P₂), which in turn activates 6-phosphofructo-1-kinase (6PFK1) to enhance glycolysis [111, 112]. This effect is involved in adipocyte lipogenesis and triglyceride synthesis [110]. PFKFB3 is decreased in adipose tissue of KKAY mice which is a model of type 2 diabetes and in PPAR γ 2-deficient mice which is a model of insulin resistance. However, it is unknown how the metabolic properties of PFKFB3/iPFK2 are related to the regulation of adipose tissue function, in particular adipose tissue inflammatory response.

MATERIALS AND METHODS

Animal Experiments

Homozygous disruption of PFKFB3/iPFK2 is embryonic lethal [133]. Thus, PFKFB3^{+/-} mice, generated as previously described [133], were used in the present study. Adipocyte-specific PFKFB3/iPFK2 overexpression mice were also generated in this study [134, 135]. Wild-type littermates (C57BL/6J background) were used as the control. All mice were maintained on a 12:12-h light-dark cycle (lights on at 06:00). At the age of 5 - 6 weeks, mice were fed an HFD (60% fat calories, 20% protein calories,

and 20% carbohydrate calories) or low-fat diet (LFD) (10% fat calories, 20% protein calories, and 70% carbohydrate calories) for 12 weeks. Both diets are products of Research Diets, Inc (New Brunswick, NJ) and contain the same amount of casein, L-cystein, cellulose, soybean oil, and minerals. However, the HFD contains much more lard and maltodextrin but much less sucrose and none corn starch compared to the LFD. During the 12-week feeding period, body weight and food intake of the mice were recorded every 4 days. After the feeding regimen, mice were fasted for 4 h before sacrifice for collection of blood and tissue samples [135-137]. After anesthesia with ketamine/Xylene (100 mg/kg / 10 mg/kg body weight) via intraperitoneal injection, the abdomen was quickly opened. Epididymal, mesenteric, and perinephric fat depots were dissected and weighed as visceral fat content [136]. After weighing, adipose tissue samples were either fixed and embedded for histological and immunohistochemical analyses or frozen in liquid nitrogen and then stored at – 80 °C for further analyses. Some mice were fasted similarly and used for insulin and glucose tolerance tests and insulin signaling analyses. All study protocols were reviewed and approved by the Institutional Animal Care and Use Committee of Texas A&M University.

Determination of PFKFB3 mRNA, iPFK2 Amount, and F26P₂ level

PFKFB3 mRNA and iPFK2 amount in the adipose tissue were determined using real-time RT-PCR and Western blot, respectively, as described below. Similarly, the amount of iPFK2 in the brown adipose and liver tissues was determined using Western blot. The levels of F26P₂ were measured using the 6PFK1 activation method [135].

Measurement of Adipose Tissue Lipolysis

The assays were conducted in the same ways as described by Berger and Haemmerle [138, 139]. Briefly, freshly isolated adipose tissue samples were washed several times with PBS and incubated in a final volume of 1 ml high glucose Dulbecco's modified Eagle's medium (DMEM) containing 2% fatty acid-free BSA with or without 10 μ M isoproterenol at 37°C for 3 h. Aliquots of the medium were collected hourly to quantify glycerol content using metabolic kits (BioVision, Mountain View, CA). Lipolysis was estimated as the efflux of glycerol.

Insulin and Glucose Tolerance Tests

Mice were fasted for 4 h and received an intraperitoneal injection of insulin (1 U/kg) or D-glucose (2 g/kg). For insulin tolerance tests, blood samples (5 μ l) were collected from the tail vein before and at 15, 30, 45, and 60 min after the bolus insulin injection. Similarly, for glucose tolerance tests, blood samples were collected from the tail vein before and at 30, 60, 90 and 120 min after the glucose bolus injection [137].

Measurement of Plasma Metabolic Parameters

The levels of plasma glucose, triglycerides, and free fatty acids were measured using metabolic assay kits (Sigma, St. Louis, MO and BioVision, Mountain View, CA). The levels of plasma insulin and leptin were measured using ELISA kits (Crystal Chem Inc., Downers Grove, IL).

Histological and Immunohistochemical Analyses of Adipose Tissue

The paraffin-embedded adipose tissue blocks were cut into sections of 5 μm thickness and stained with H&E. In addition, the sections were stained for the expression of F4/80 with rabbit anti-F4/80 (1:100) (AbD Serotec, Raleigh, NC) as previously described [125, 140]. The fraction of F4/80-expressing cells for each sample is calculated as the sum of the number of nuclei of F4/80-expressing cells divided by the total number of nuclei in sections of a sample. Six fields per slide were included, and a total of 4 to 6 mice per group were used.

Isolation of Stromal Vascular Cells (Macrophages) and Adipocytes from Adipose Tissue

Stromal vascular cells (SVCs) and adipocytes were isolated using the collagenase digestion method as previously described [97]. After digestion and centrifugation, the pelleted cells were collected as SVCs and the floating cells were harvested as adipocytes.

Cell Culture and Treatment

3T3-L1 cells were maintained in high glucose DMEM supplemented with 10% fetal bovine serum (FBS), 100U/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin. To differentiate 3T3-L1 cells, the 2 d post-confluent cells were incubated in growth medium supplemented with 10 $\mu\text{g}/\text{ml}$ insulin, 1 μM dexamethasone, and 0.5 mM 3-isobutyl-1-

methyl-xanthine for 48 h, followed by incubation for an additional 6 – 8 d in growth medium supplemented with 10 g/ml insulin. To knock down PFKFB3/iPFK2, predifferentiated 3T3-L1 cells were transfected with the plasmid containing shRNA against mouse PFKFB3 (iPFK2-KD) (OriGene, Rockville, MD) with Lipofectamine™ 2000 Transfection Reagent (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. Similarly, predifferentiated 3T3-L1 cells were transfected with shRNA vector (iPFK2-Ctrl) and served as the control. To overexpress PFKFB3/iPFK2, predifferentiated 3T3-L1 cells were transfected with plasmid containing the cDNA of iPFK2. As a control, predifferentiated 3T3-L1 cells were transfected with empty vector. After transfection for 24 h, the cells were induced for differentiation for 6 – 8 d. Thereafter, iPFK2-KD adipocytes, iPFK2-OX adipocytes, two iPFK2-Ctrl adipocytes, as well as un-transfected adipocytes were used for the following assays. Some transfected predifferentiated 3T3-L1 cells were selected with puromycin (5 µg/ml) to establish stable cell lines for further analyses.

To verify PFKFB3/iPFK2 knockdown or overexpression, cell lysates were prepared and used to determine iPFK2 amount using Western blot. To determine the rate of glucose incorporation into lipid, each well (6-well plate) of the cells was incubated with DMEM supplemented with 1 µCi [U-¹⁴C]-glucose for 24 h as previously described [141]. After sequential extraction with 30% KOH, 95% ethanol, 9 M H₂SO₄, and petroleum ether, the amount of [¹⁴C]-lipids was quantified using a Beckman liquid scintillation counter. To quantify adipocyte lipid content, the cells were stained with Oil Red O. The lipid-associated dye was extracted with isopropanol for 15 min. The optical

density (OD) of the extraction solution was measured using spectrophotometer at 510 nm [120]. To determine the status of adipocyte oxidative stress, the cells were treated with palmitate (250 μ M) or vehicle for 24 h and used to measure the production of reactive oxygen species (ROS) using the nitroblue tetrazolium (NBT) assay as previously described [142]. Additionally, after differentiation, stable adipocytes were treated with or without palmitate (250 μ M) for 24 h. In the last hour of treatment, 100 μ M etomoxir (an inhibitor of carnitine palmitoyltransferase-1, CPT1) or 10 mM N-acetyl-L-cysteine (an antioxidant) was added, and the production of ROS was determined. To determine adipocyte expression of adipokines and proinflammatory cytokines, the total RNA of the cells was prepared and used for real-time RT-PCR. To determine changes in inflammatory signaling, the cells were treated with or without lipopolysaccharides (LPS, 100 ng/ml) for 1 h prior to harvest. Cell lysates were prepared and used to measure the levels and phosphorylation states of JNK and NF κ B p65 using Western blots. To determine changes in insulin signaling, the cells were treated with or without insulin (100 nM) for 30 min prior to harvest. Cell lysates were prepared and used to measure the levels and phosphorylation state of Akt using Western blots.

RNA Isolation, Reverse Transcription, Real-time PCR, and Microarray

The total RNA was isolated from frozen tissue samples and cultured/isolated cells. RNA isolation and real-time RT-PCR were conducted as previously described [137]. The mRNA levels were analyzed for PFKFB3, F4/80, TNF α , IL-6, resistin, adiponectin, peroxisome proliferator-activated receptor alpha (PPAR α), PPAR gamma

co-activator 1 (PGC1), and Carnitine palmitoyltransferase I (CPT1) in adipose tissue samples and/or cell samples. Additionally, the RNA of adipose tissue samples from HFD-fed PFKFB3^{+/-} and wild-type littermates were used for mouse Agilent 44K microarray using methods as previously described [143, 144].

Western Blots

Lysates were prepared from frozen tissue samples and cultured cells. Western blots were conducted as previously described [136, 137]. The levels of iPFK2, Akt1/2, phospho-Akt (Ser473), JNK, phospho-JNK, NFκB p65 and phospho-p65 were analyzed.

Statistical Methods

Numeric data are presented as means ± SE (standard error). Statistical significance was assessed by unpaired, two-tailed ANOVA or Student's *t* test. Differences were considered significant at the two-tailed $P < 0.05$.

RESULTS OF ANIMAL STUDIES

The Distribution of PFKFB3/iPFK2

The expression profile of PFKFB3/iPFK2 was determined in various tissues in wild-type mice. Among the key tissues that are involved in the regulation of systemic insulin sensitivity and metabolic homeostasis, PFKFB3/iPFK2 is expressed at high levels in the adipose tissue and expressed at very low levels in the liver and muscle

(Fig.8). These data suggest that PFKFB3/iPFK2-associated metabolic changes are due primarily to alteration of PFKFB3/iPFK2 in the adipose tissue.

The Confirmation of PFKFB3^{+/-} Mice

Homozygous disruption of PFKFB3/iPFK2 is embryonic lethal [133]. Thus, PFKFB3^{+/-} mice were generated for the present study. Disruption of PFKFB3/iPFK2 was confirmed using PCR (Fig. 9).

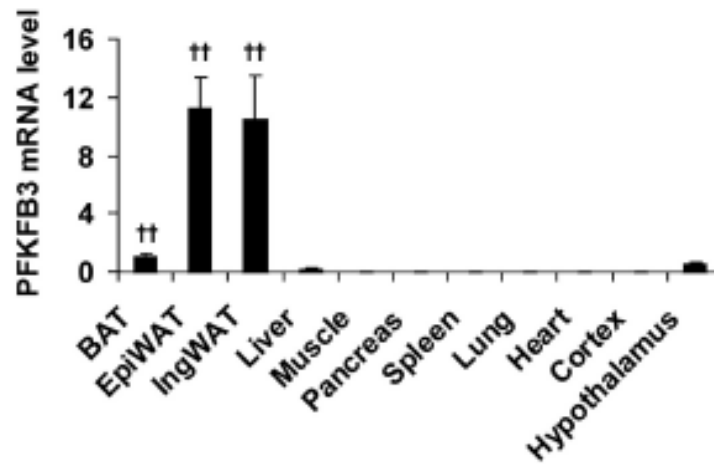


Figure 8. The Distribution of PFKFB3/iPFK2. Wild-type C57BL/6J mice were used for analyses. PFKFB3 is abundantly expressed in epididymal (Epi) and inguinal (Ing) white adipose tissue. BAT, brown adipose tissue. Data are means \pm SE, $n = 4$. †† $P < 0.01$ vs. liver.

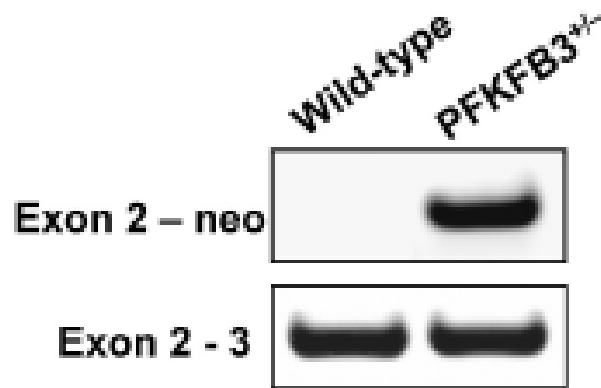


Figure 9. The Confirmation of PFKFB3^{+/-} Mice. PCR analyses of mouse genomic DNA using an exon 2-specific primer with a neomycin-specific primer (+/-, heterozygous) or an exon 3-specific primer (+/+, wild-type).

Changes of PFKFB3 mRNA and iPFK2 Amount and Activity in PFKFB3^{+/-} Mice

PFKFB3^{+/-} mice were used to examine changes in PFKFB3 mRNA and iPFK2 amount and activity in the adipose tissue. Disruption of PFKFB3/iPFK2 resulted in decreased PFKFB3/iPFK2 expression at both the mRNA and protein levels (Fig. 10 A, B and C). Compared to that in epididymal adipose tissue, the amount of iPFK2 was less abundant in the brown adipose tissue (Fig.10 B and C). Because PFKFB3/iPFK2 determines the production of F26P₂, the levels of adipose tissue F26P₂ were quantified as a direct indicator of iPFK2 activity. In PFKFB3^{+/-} mice, the levels of adipose tissue F26P₂ were significantly lower than those in wild-type littermates (Fig. 10 D).

Disruption of PFKFB3/iPFK2 Blunts HFD-induced Weight Gain

Feeding an HFD to mice induces obesity [115, 124]. To determine the effect of PFKFB3/iPFK2 disruption on diet-induced obesity, PFKFB3^{+/-} mice and wild-type littermates were fed on an HFD. As the control, PFKFB3^{+/-} mice and wild-type littermates were fed an LFD. Following the diet feeding for a period of 12 weeks, PFKFB3^{+/-} mice weighed only slightly lesser than wild-type littermates on an LFD. However, on an HFD, PFKFB3^{+/-} mice exhibited a much smaller gain in body weight than did wild-type littermates (Fig. 11).

Disruption of PFKFB3/iPFK2 Blunts HFD-induced Adiposity

HFD-fed PFKFB3^{+/-} mice exhibited a much smaller gain in body weight than did wild-type littermates. This was attributed, at least in part, to a smaller increase in visceral fat content (Fig. 12 A and B). Consistently, the adipocytes in PFKFB3^{+/-} mice were markedly less enlarged than in wild-type littermates in response to HFD feeding as determined by histology (Fig. 12 C). To determine the possible contribution of food intake to the difference in body weight and adiposity, food intake of the mice was also monitored. However, there was no difference in food intake (data not shown).

Disruption of PFKFB3/iPFK2 Alters Adipose Tissue Lipolysis

Feeding an HFD to mice leads to adipose tissue dysfunction. To determine the effect of PFKFB3/iPFK2 disruption on HFD-induced adipose tissue dysfunction,

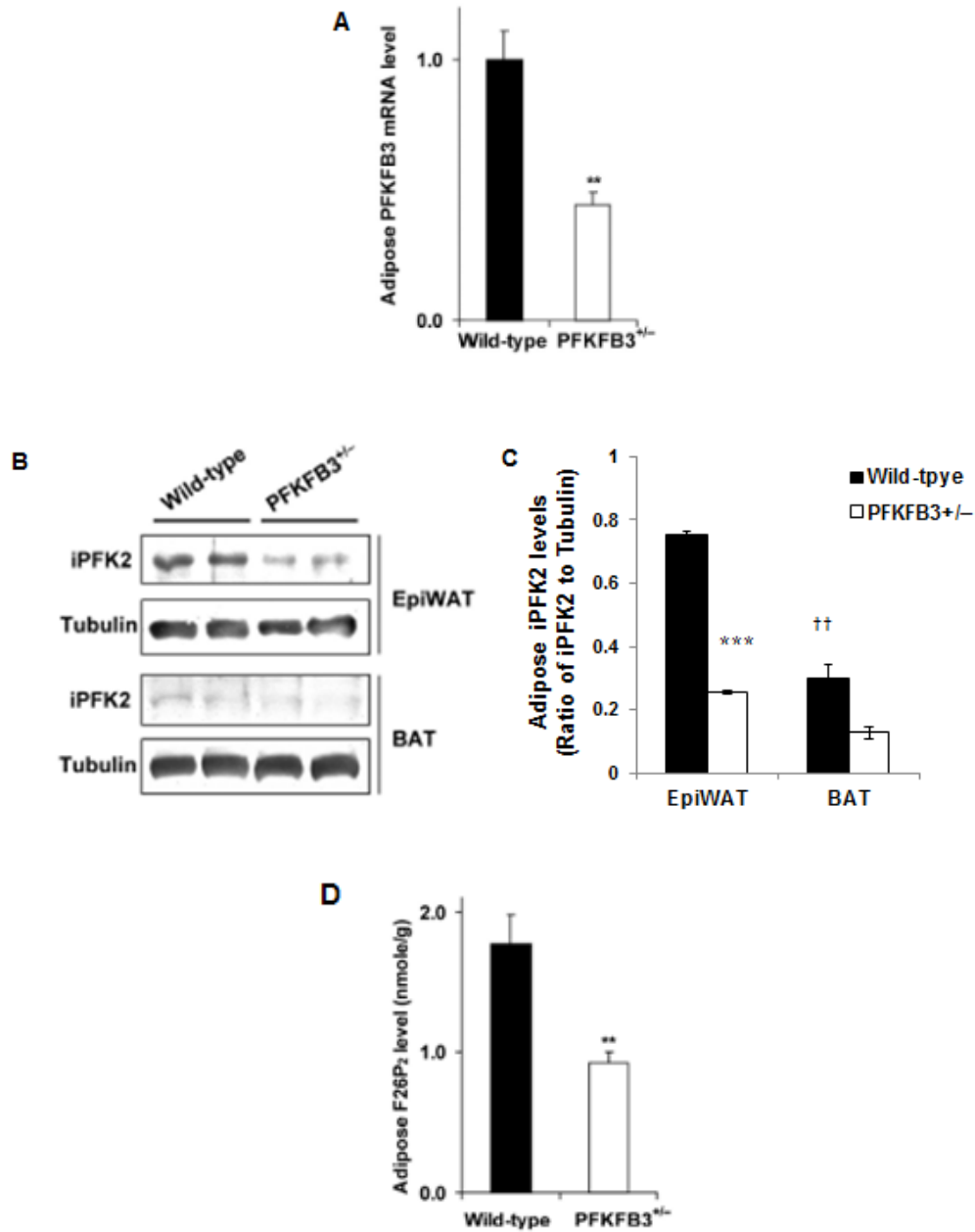


Figure 10. Changes of PFKFB3 mRNA and iPFK2 Amount and Activity in PFKFB3^{+/-} Mice. A, The levels of PFKFB3 mRNA in epididymal adipose tissue were quantified using real-time RT-PCR. B, The amount of iPFK2 in both epididymal adipose tissue and brown adipose tissue was measured using Western blot. C, The levels of iPFK2 was quantified. D, The levels of F26P₂ in epididymal adipose tissue were determined using the 6PFK1 activation method. For A, C and D, data are means \pm SE, $n = 2 - 6$. ** $P < 0.01$ and *** $P < 0.001$ PFKFB3^{+/-} vs. wild-type; †† $P < 0.01$ wild-type EpiWAT vs. wild-type BAT.

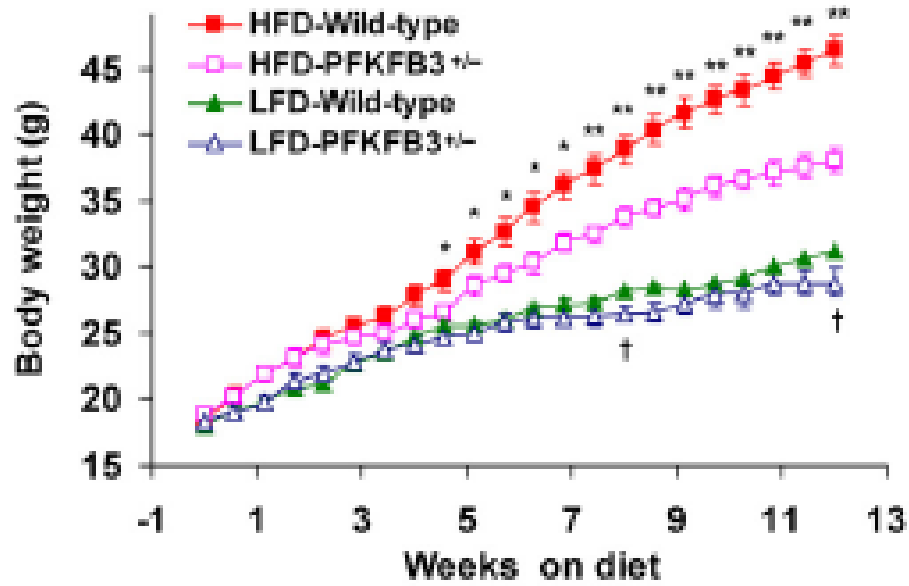


Figure 11. Disruption of PFKFB3/iPFK2 Blunts HFD-induced Weight Gain. At the age of 5 – 6 weeks, PFKFB3^{+/-} mice and wild-type littermates were fed a HFD or LFD for 12 weeks. Data are means \pm SE, n = 6. * $P < 0.05$ and ** $P < 0.01$ HFD-PFKFB3^{+/-} vs. HFD-wild-type; † $P < 0.05$ LFD-PFKFB3^{+/-} vs. LFD-wild-type.

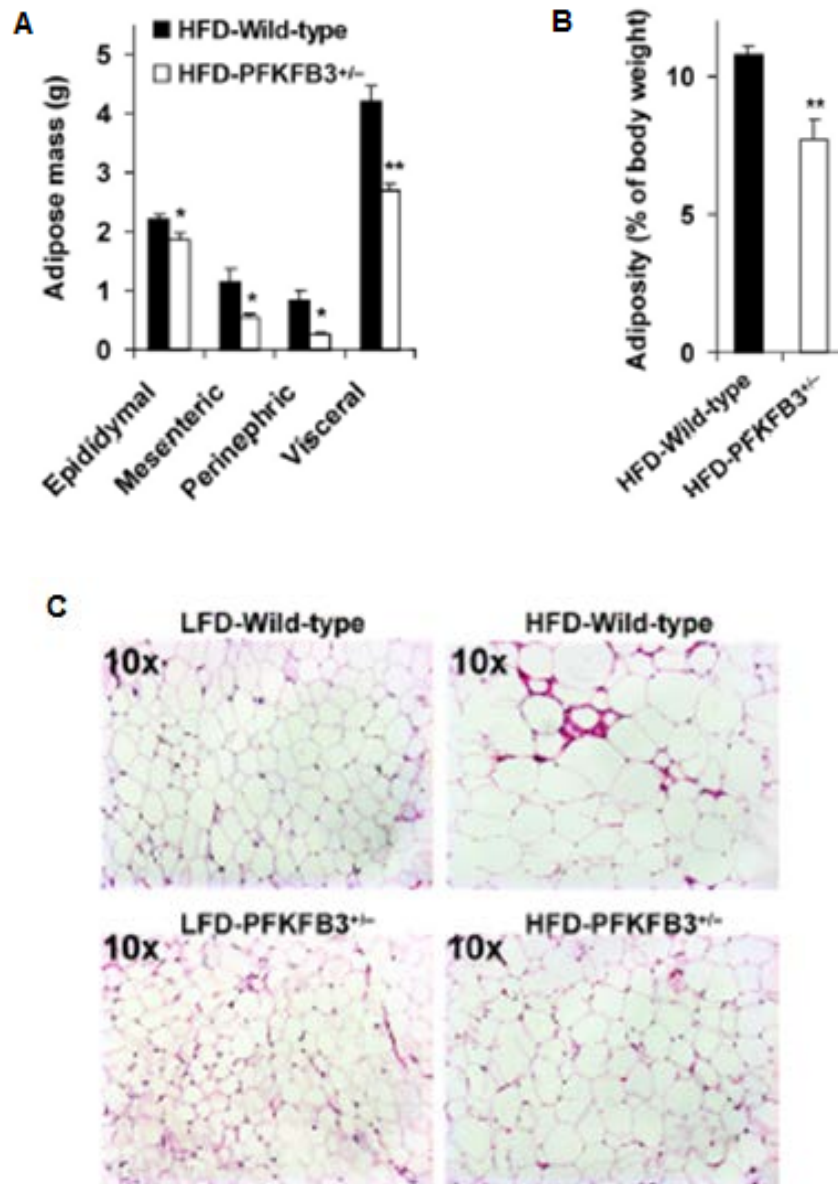


Figure 12. Disruption of PFKFB3/iPFK2 Blunts HFD-induced Adiposity. At the age of 5 – 6 weeks, PFKFB3^{+/-} mice and wild-type littermates were fed a HFD or LFD for 12 weeks. A and B, Changes in visceral fat content. The sum of epididymal, mesenteric, and perinephric fat mass was estimated as visceral fat content. C, Adipose tissue histology. The sections of epididymal fat pad were stained with H&E. For A and B, data are means \pm SE, n = 6. * $P < 0.05$ and ** $P < 0.01$ HFD-PFKFB3^{+/-} vs. HFD-wild-type.

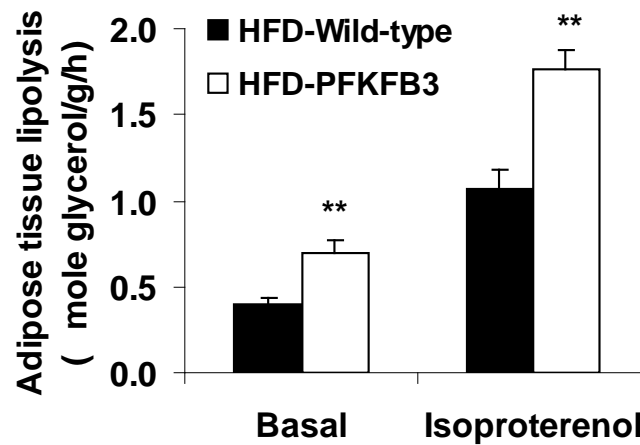


Figure 13. Disruption of PFKFB3/iPFK2 Alters Adipose Tissue Lipolysis. At the age of 5 – 6 weeks, PFKFB3^{+/-} mice and wild-type littermates were fed a HFD for 12 weeks. The rates of adipose tissue lipolysis were measured under both basal and isoproterenol-stimulated conditions. Data are means \pm SE, n = 6. * $P < 0.05$ and ** $P < 0.01$ HFD-PFKFB3^{+/-} vs. HFD-wild-type.

changes in adipose tissue lipolysis was analyzed. Compared to those in HFD-fed wild-type littermates, the rates of adipose tissue lipolysis were significantly higher in HFD-fed PFKFB3^{+/-} mice under both basal and isoproterenol-stimulated conditions (Fig. 13).

Disruption of PFKFB3/iPFK2 Alters Adipokine Expression

To determine the effect of PFKFB3/iPFK2 disruption on HFD-induced adipose tissue dysfunction, changes in adipokine expression was analyzed. The mRNA levels of resistin were increased and the mRNA levels of adiponectin were decreased in the

adipose tissue in HFD-fed PFKFB3^{+/-} mice compared to their respective levels in HFD-fed wild-type littermates (Fig. 14).

The Effects of Disruption of PFKFB3/iPFK2 on Macrophage Infiltration

Feeding an HFD to mice induces adipose tissue inflammation, which is characterized by macrophage infiltration and increased proinflammatory cytokine expression [125, 126]. The effects of disruption of PFKFB3/iPFK2 on macrophage infiltration were measured. Compared to LFD-fed mice, HFD-fed PFKFB3^{+/-} mice and wild-type littermates both revealed macrophage infiltration into the adipose tissue (data of LFD-fed mice not shown). However, PFKFB3^{+/-} mice exhibited a smaller increase in HFD-induced macrophage infiltration in the adipose tissue compared to wild-type littermates (Fig. 15 A and B). Additionally, HFD feeding did not increase adipose tissue mRNA levels of F4/80 in PFKFB3^{+/-} mice as did in wild-type mice (Fig. 15 C), which is consistent with changes in macrophage infiltration in the adipose tissue.

Disruption of PFKFB3/iPFK2 Exacerbates HFD-induced Adipose Tissue

Inflammatory Response

Feeding an HFD to mice induces adipose tissue inflammation, which is characterized by macrophage infiltration and increased proinflammatory cytokine expression [125, 126]. PFKFB3^{+/-} mice exhibited a smaller increase in macrophage infiltration compared to HFD-fed wild-type mice. However, the mRNA levels of TNF α

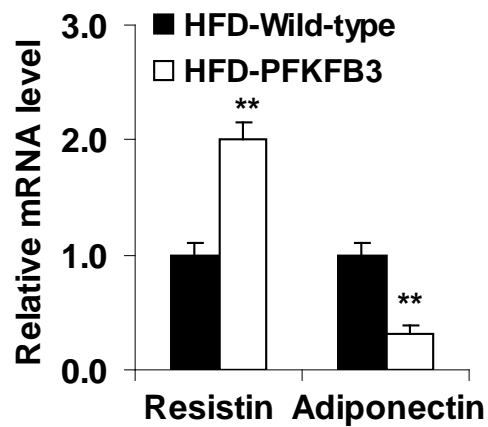


Figure 14. Disruption of PFKFB3/iPFK2 Alters Adipokine Expression. At the age of 5 – 6 weeks, PFKFB3^{+/-} mice and wild-type littermates were fed a HFD for 12 weeks. The mRNA levels of adipose tissue resistin and adiponectin were measured. Data are means \pm SE, n = 6. * $P < 0.05$ and ** $P < 0.01$ HFD-PFKFB3^{+/-} vs. HFD-wild-type.

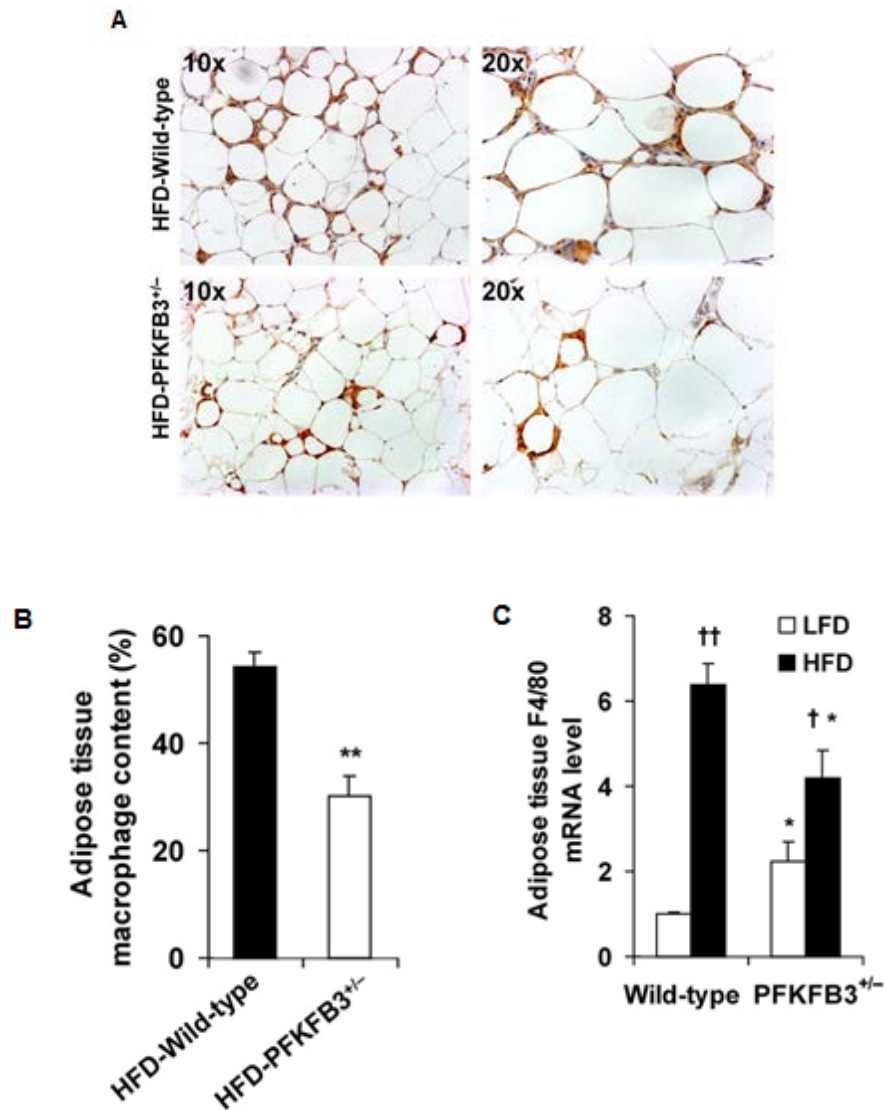


Figure 15. The Effects of Disruption of PFKFB3/iPFK2 on Macrophage Infiltration. At the age of 5 – 6 weeks, PFKFB3^{+/-} mice and wild-type littermates were fed a HFD for 12 weeks. A, Macrophage infiltration in adipose tissue. The sections of epididymal fat pad were immunostained for F4/80. B, Fraction of adipose tissue macrophages. C, The mRNA levels of adipose tissue F4/80. Data are means \pm SE, $n = 4 - 6$. * $P < 0.05$ and ** $P < 0.01$ HFD-PFKFB3^{+/-} vs. HFD-wild-type or PFKFB3^{+/-} vs. wild-type on the same diet; † $P < 0.05$ and †† $P < 0.01$ HFD vs. LFD for the same genotype.

and IL-6 in adipose tissue were higher in HFD-fed PFKFB3^{+/-} mice than in HFD-fed wild-type littermates (Fig. 16 A). Furthermore, similar trends were also observed in the mRNA levels of TNF α and IL-6 in macrophages and adipocytes isolated from the adipose tissue (Fig. 16 B and C). These data suggest that disruption of PFKFB3/iPFK2 increases HFD-induced adipose tissue inflammatory response although it causes less macrophage infiltration.

Disruption of PFKFB3/iPFK2 Increases Adipose Expression of Genes Involved in Fatty Acid Oxidation

To explore the role played by PFKFB3/iPFK2 in linking nutrient metabolism and the inflammatory response, the adipose tissue of HFD-fed mice was used for microarray analyses. Compared to wild-type littermates, PFKFB3^{+/-} mice exhibited changes in the expression of 495 genes from a total of 23694 genes ($P < 0.01$). Of interest, PFKFB3^{+/-} mice showed an increase in the expression of PPAR α and PGC1, two master regulators that stimulate fatty acid oxidation [145-148], as well as an increase in the expression of oxidative-stress responsive 1 (Table 1). Using real-time RT-PCR, the increase in the mRNA levels of PPAR α and PGC1 was confirmed (Fig. 17). Furthermore, the mRNA levels of CPT1, a key enzyme that controls the rate-limiting step of fatty acid oxidation [149, 150], was markedly increased in PFKFB3^{+/-} mice (Fig. 17). These data suggest that PFKFB3/iPFK2 controls the status of adipocyte oxidative stress by regulating the balance between glycolysis and fatty acid oxidation.

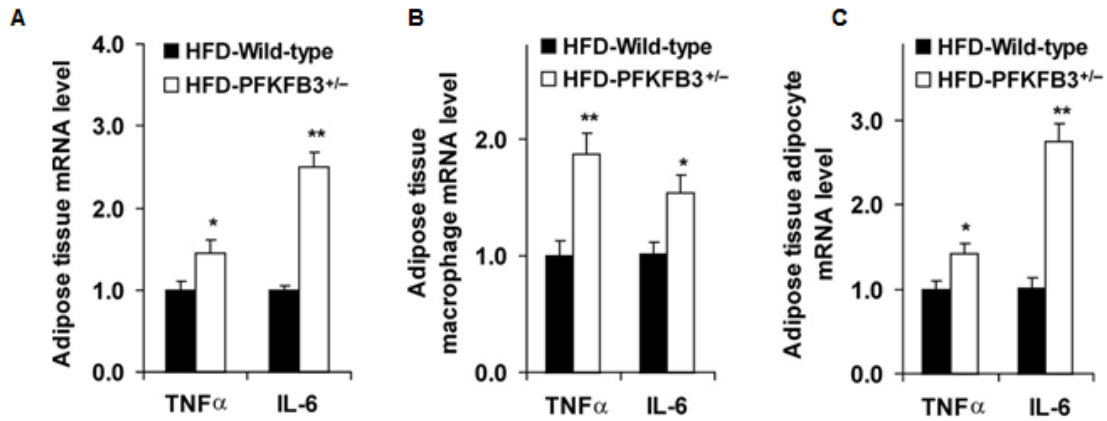


Figure 16. Disruption of PFKFB3/iPKF2 Exacerbates HFD-induced Adipose Tissue Inflammatory Response. At the age of 5 – 6 weeks, PFKFB3^{+/-} mice and wild-type littermates were fed a HFD for 12 weeks. The mRNA levels of inflammatory markers were quantified using real-time RT-PCR. A-C, The mRNA levels of TNF α and IL-6 in adipose tissue (A), as well as in macrophages (B) and adipocytes (C) isolated from adipose tissue. Data are means \pm SE, n = 4 – 6. * $P < 0.05$ and ** $P < 0.01$ HFD-PFKFB3^{+/-} vs. HFD-wild-type.

Table 1. Selected adipose genes that are altered by disruption of PFKFB3/iPFK2

<i>Gene symbol</i>	<i>Gene name</i>	<i>Fold of change</i>
Gcgr	Glucagon receptor	2.56 *
Retn	Resistin	2.15 *
Ppara	Peroxisome proliferator activated receptor alpha	1.68 *
Ptprb	Protein tyrosine phosphatase, receptor type, B	1.63 *
Ppargc1a	Peroxisome proliferative activated receptor, gamma, coactivator 1 alpha	1.56 *
Rbp4	Retinol binding protein 4	1.54 *
Ptprm	Protein tyrosine phosphatase, receptor type, M	1.52 *
	Oxidative-stress responsive 1	1.24 *
Tnfrsf1b	Tumor necrosis factor receptor superfamily, member 1b	0.67 *
Stat3	Signal transducer and activator of transcription 3	0.66 *
Il1rn	Interleukin 1 receptor antagonist	0.65 *
Srxn1	Sulfiredoxin 1 homolog (<i>Saccharomyces. cerevisiae</i>)	0.64 *
Tnfrsf11a	Tumor necrosis factor receptor superfamily, member 11a	0.60 *
Acsl4	Acyl-CoA synthetase long-chain family member 4	0.60 *
Timp1	Tissue inhibitor of metalloproteinase 1	0.44 *

Adipose mRNA transcriptome was analyzed in HFD-fed PFKFB3^{+/-} mice and wild-type littermates using microarray approach. Changes in the mRNA levels of adipose genes were expressed as the PFKFB3^{+/-}/wild-type ratios. n = 4. * $P < 0.05$ PFKFB3^{+/-} vs. wild-type.

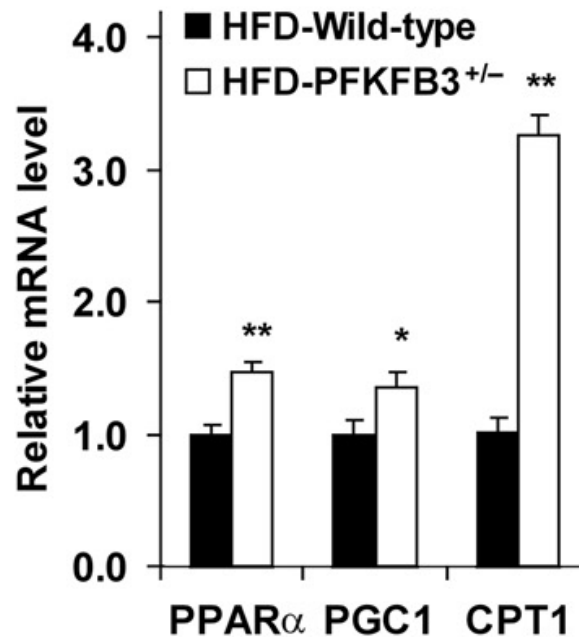


Figure 17. Disruption of PFKFB3/iPFK2 increases adipose expression of genes involved in fatty acid oxidation. At the age of 5 – 6 weeks, PFKFB3^{+/-} mice and wild-type littermates were fed a HFD for 12 weeks. The mRNA levels of genes involved in fatty acid oxidation in adipose tissue were quantified using real-time RT-PCR. Data are means \pm SE, n = 4 – 6. * $P < 0.05$ and ** $P < 0.01$ HFD-PFKFB3^{+/-} vs. HFD-wild-type.

Disruption of PFKFB3/iPFK2 Decreases Adipose Tissue Insulin Signaling

To determine the effect of PFKFB3/iPFK2 disruption on HFD-induced adipose tissue dysfunction, changes adipose tissue insulin signaling were analyzed. Compared to HFD-fed wild-type mice, HFD-fed PFKFB3^{+/-} mice exhibited a marked decrease in insulin-stimulated Akt phosphorylation (Fig. 18 A and B), indicating exacerbation of adipose tissue insulin resistance.

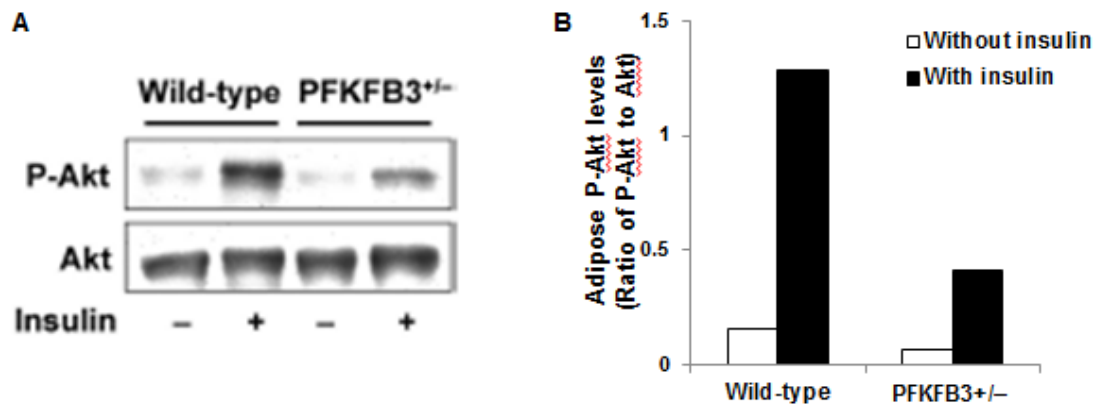


Figure 18. Disruption of PFKFB3/iPFK2 changes adipose tissue insulin signaling. At the age of 5 – 6 weeks, PFKFB3^{+/-} mice and wild-type littermates were fed a HFD for 12 weeks. . After anesthesia by an intraperitoneal (i.p.) injection of pentobarbital (50 mg/kg body weight), mice were injected with insulin (1 U/kg) or PBS into the inferior vena cava (i.v.) and epididymal adipose tissue samples were collected 5 min later. A, Adipose tissue insulin signaling was determined by using western blot. B. The P-Akt and Akt levels were quantified.

Disruption of PFKFB3/iPFK2 Exacerbates HFD-induced Systemic Insulin Resistance

Disruption of PFKFB3/iPFK2 changed adipose tissue lipolysis and adipokine expression, increased adipose tissue inflammatory response, and impaired adipose tissue insulin signaling, suggesting that disruption of PFKFB3/iPFK2 increases the severity of HFD-induced adipose tissue dysfunction. Adipose tissue dysfunction can contribute to the development of systemic insulin resistance [151, 152]. To determine changes in systemic insulin sensitivity, insulin and glucose tolerance tests were conducted. On a HFD, insulin resistance and glucose intolerance were more severe in PFKFB3^{+/-} mice

than in wild-type littermates (Fig. 19 A and B), which were correlated with adipose tissue dysfunction well. Additionally, the levels of plasma glucose were significantly higher in PFKFB3^{+/-} mice than in wild-type littermates on both an LFD and HFD (Table 2). Consistent with changes in adiposity, the levels of plasma leptin were lower in PFKFB3^{+/-} mice than in wild-type littermates on an HFD (Table 2). This may also contribute to exacerbation of insulin resistance in PFKFB3^{+/-} mice.

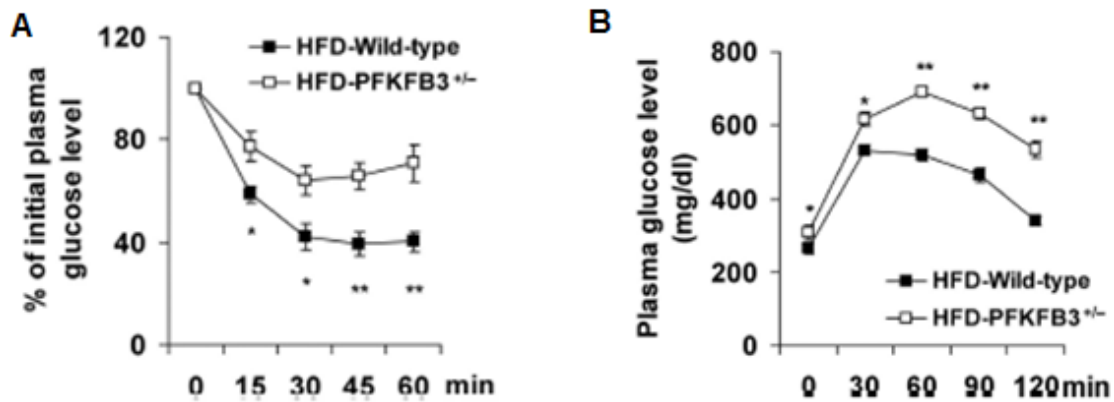


Figure 19. Disruption of PFKFB3/iPFK2 exacerbates HFD-induced systemic insulin resistance. At the age of 5 – 6 weeks, PFKFB3^{+/-} mice and wild-type littermates were fed a HFD for 12 weeks. For A and B, mice were fasted for 4 h and received an i.p. injection of insulin (1 U/kg) (A) or D-glucose (2 g/kg) (B). Data are means \pm SE, n = 6. * $P < 0.05$ and ** $P < 0.01$ HFD-PFKFB3^{+/-} vs. HFD-wild-type.

Selective PFKFB3/iPFK2 Overexpression in Adipose Tissue

To address a direct role for the PFKFB3/iPFK2 in adipocytes, Tg mice was generated for analyzing aspects of diet-induced inflammatory and metabolic responses (Fig. 20 A and B). In confirmatory experiments, PFKFB3/iPFK2 overexpression was targeted to adipose tissue, but not in liver, muscle, or bone marrow (Fig. 20 C). Thus, Tg

Table 2. Changes in the levels of plasma metabolites and insulin

<i>Serum Factor</i>	<i>Wild-type</i>	<i>PFKFB3^{+/-}</i>
Glucose (mg/dl)		
LFD	156 ± 16	202 ± 11 *
HFD	238 ± 6 ^{††}	308 ± 8 ** ^{††}
Insulin (ng/ml)		
LFD	2.1 ± 0.3	3.2 ± 1.3
HFD	5.3 ± 1.6 [†]	8.1 ± 0.8 * ^{††}
Leptin (ng/ml)		
LFD	4.2 ± 1.3	2.0 ± 0.7
HFD	65.0 ± 6.6 ^{††}	43.8 ± 3.7 * ^{††}
Free fatty acids (mM)		
LFD	0.1 ± 0	0.2 ± 0 *
HFD	0.2 ± 0.1 [†]	0.3 ± 0 [†]
Triglycerides (mg/dl)		
LFD	34 ± 1	40 ± 3 *
HFD	48 ± 4 [†]	51 ± 2 [†]

At the age of 5 – 6 weeks, PFKFB3^{+/-} mice and wild-type littermates were fed a HFD or LFD for 12 weeks. After the feeding regimen, mice were fasted for 4 h before collection of plasma samples. Data are means ± SE, n = 6. * $P < 0.05$ and ** $P < 0.01$ PFKFB3^{+/-} vs. wild-type on the same diet; [†] $P < 0.05$ and ^{††} $P < 0.01$ HFD vs. LFD for the same genotype.

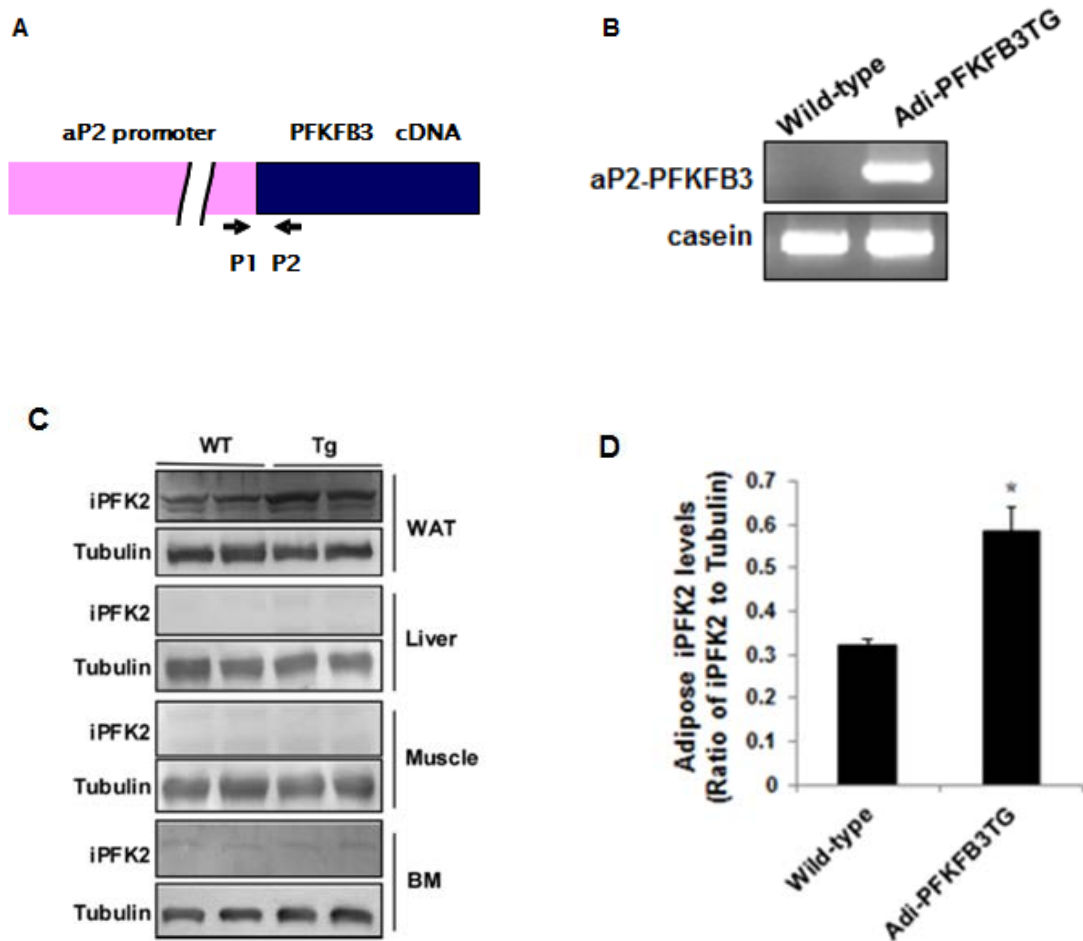


Figure 20. The generation of PFKFB3/iPFK2 adipocyte-specific overexpression mice. A. PCR analyses of mouse genomic DNA using an exon 2-specific primer with a neomycin-specific primer (+/–, heterozygous) or an exon 3-specific primer (+/+, wild-type). B, genomic DNA was prepared from Adi-PFKFB3TG (Tg) mice and wild-type littermates and used for PCR analyses of aP2-PFKFB3 transgene. C. iPFK2 (encoded by PFKFB3) abundance in white adipose tissue (WAT), liver, skeletal muscle, and bone marrow (BM) was measured using Western blots. D, The levels of F26P₂ in epididymal adipose tissue were determined using the 6PFK1 activation method. For D, data are means ± SE, n = 2 – 6. * $P < 0.05$ PFKFB3^{+/-} vs. wild-type.

mice used here in overexpressed PFKFB3 selectively in adipose tissue. Because PFKFB3/iPFK2 determines the production of F26P₂, the levels of adipose tissue F26P₂ were quantified as a direct indicator of iPFK2 activity. In Tg mice, the levels of adipose tissue F26P₂ were significantly higher than those in wild-type littermates (Fig. 20 D).

Adipocyte-specific PFKFB3/iPFK2 Overexpression Increases Body Weight and Adiposity

To determine the effect of PFKFB3/iPFK2 overexpression on diet-induced obesity, Tg mice and wild-type littermates were started on an HFD. As the control, Tg mice and wild-type littermates were fed an LFD. Following the diet feeding for a period of 12 weeks, the weight and fat mass of Tg mice are slightly higher than wild-type littermates on an LFD and an HFD (Fig. 21 A and B).

Adipocyte-specific PFKFB3/iPFK2 Overexpression Protects Against Overnutrition-induced Insulin Resistance and Glucose Intolerance

Although the weight and fat mass of Tg mice are slightly higher than wild-type littermates on an HFD, Tg mice displayed a much smaller increase in the severity of HFD induced systemic insulin resistance and glucose intolerance (Fig. 22 A and B). Therefore, selective overexpression of PFKFB3/iPFK2 in adipose tissue protects mice from diet-induced systemic insulin resistance and metabolic dysregulation.

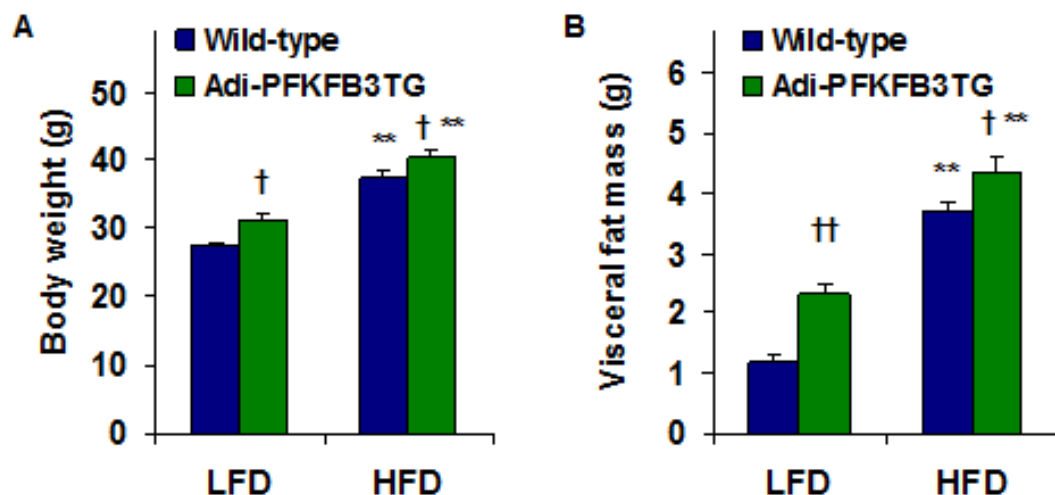


Figure 21. Adipocyte-specific PFKFB3/iPFK2 overexpression increases body weight and adiposity. At the age of 5 – 6 weeks, Tg mice and wild-type littermates were fed a HFD or LFD for 12 weeks. A and B, Changes in weight and fat mass. For A and B, data are means \pm SE, $n = 6$. $** P < 0.01$ HFD vs. LFD for the same genotype. $^{\dagger} P < 0.05$ and $^{\dagger\dagger} P < 0.01$ wild type mice vs. Tg mice.

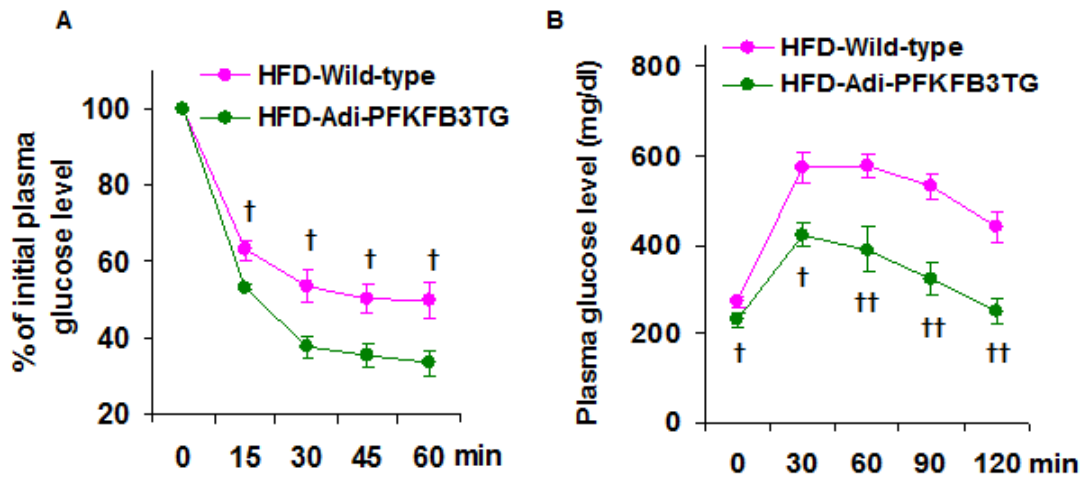


Figure 22. Adipocyte-specific PFKFB3/iPFK2 overexpression protects against overnutrition-induced insulin resistance and glucose intolerance. A, insulin tolerance tests. B, glucose tolerance tests. A and B, male Tg mice and WT mice, at 5–6 weeks of age, were fed an HFD for 12 weeks. After the feeding regimen, HFD-fed mice were fasted for 4 h and received an intraperitoneal injection of insulin (1 unit/kg) (A) or glucose (2 g/kg) (B). Data are means \pm S.E., $n = 6$ –10. †, $p < 0.05$; ††, $p < 0.01$ Tg vs. WT at the same time point.

Adipocyte-specific PFKFB3/iPFK2 Overexpression Suppresses Overnutrition-induced Adipose Tissue Inflammatory Response and Improve Insulin Sensitivity

Feeding an HFD to mice induces adipose tissue inflammation, which is characterized by macrophage infiltration and increased proinflammatory cytokine expression [125, 126]. This is considered to be critical factors contributing to systemic insulin resistance. Compared with controls, adipose tissue of HFD-fed Tg mice accumulated more macrophages (Fig. 23, A and B) but showed a significant decrease in NF- κ B p65 (Ser-468) phosphorylation (Fig. 23 C and D), indicating a decrease in

adipose tissue inflammatory response. In parallel, adipose tissue expression of proinflammatory cytokines TNF α and IL-6 was decreased (Fig. 23 E). When insulin signaling was analyzed, insulin-stimulated Akt (Ser-473) phosphorylation was increased in adipose tissue of Tg mice compared with WTmice (Fig. 23 F). Together, these results suggest that overexpression of PFKFB3/iPFK2 in adipose tissue suppresses diet-induced adipose tissue inflammatory response and insulin resistance while promoting fat deposition in adipose tissue.

RESULTS OF CELL CULTURE STUDIES

Knockdown of PFKFB3/iPFK2 Decreases Adipocyte Lipid Accumulation by Suppressing the Conversion of Glucose into Lipid

The direct role of PFKFB3/iPFK2 in regulating adipocyte metabolic and inflammatory responses was explored in 3T3-L1 adipocytes that were treated with shRNA against PFKFB3. As expected, knockdown of PFKFB3/iPFK2 was evidenced by a decrease in iPFK2 amount (Fig. 24 A and B). This resulted in a decrease in the adipocyte lipid accumulation compared to iPFK2-Ctrl adipocytes (Fig. 24 C) by suppressing the incorporation of glucose into lipid (Fig. 24 D). This results indicate that knockdown of PFKFB3/iPFK2 decreases glycolysis and glycolysis-derived lipogenesis and triglyceride synthesis.

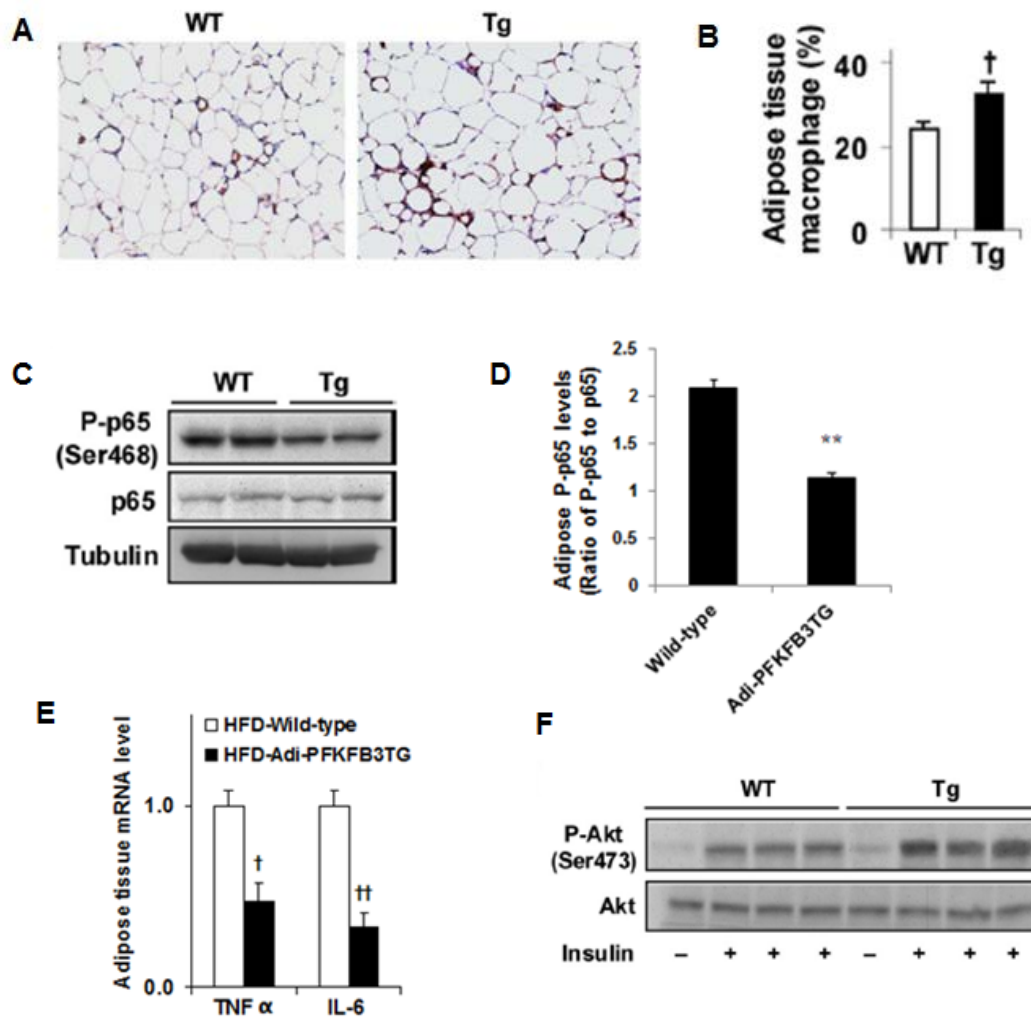


Figure 23. Adipocyte-specific PFKFB3/iPFK2 overexpression suppresses overnutrition-induced adipose tissue inflammatory response and increase insulin sensitivity. At 5–6 weeks of age, male Tg and WT mice were fed an HFD for 12 weeks. A, macrophage infiltration in adipose tissue. The sections of epididymal fat pad were immunostained for F4/80. B, fraction of adipose tissue macrophages. C and D, adipose tissue inflammatory signaling. The levels of NF- κ B p65 and phospho-p65 (Ser-468) were examined using Western blot analyses. E, TNF α and IL-6 expression were quantified using real time RT-PCR. F, adipose tissue insulin signaling. Tissue samples of HFD-fed mice were collected at 5 min after a bolus injection of insulin (1 unit/kg) or PBS into the portal vein. The levels of Akt1/2 and phospho-Akt (Ser-473) were examined using Western blot analyses. B, D and E data are means \pm S.E., $n = 6$ –10. [†], $p < 0.05$; ^{††}, $p < 0.01$ Tg vs. WT (B and E); ^{**}, $p < 0.01$ Tg vs. WT (D).

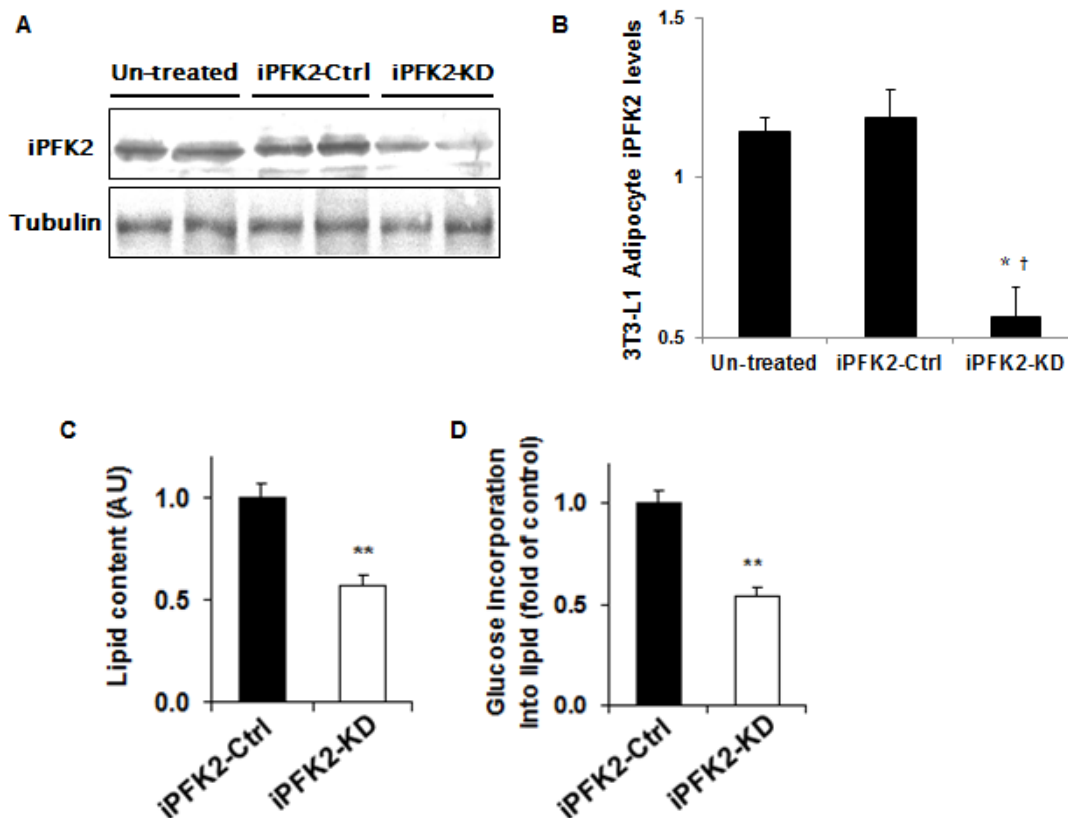


Figure 24. . Knockdown of PFKFB3/iPFK2 decreases adipocyte lipid accumulation by suppressing the conversion of glucose into lipid. Predifferentiated 3T3-L1 cells were transfected with the plasmid containing shRNA against PFKFB3/iPFK2 (iPFK2-KD) or shRNA vector (iPFK2-Ctrl) for 24 h and induced for differentiation for 6 – 8 d. Un-transfected cells were differentiated similarly and used as the un-treated control. Some transfected 3T3-L1 cells were selected by puromycin (5 μ g/ml) to establish stable iPFK2-KD or iPFK2-Ctrl cell lines. A, Western blot for iPFK2. B, The levels of iPFK2 were quantified. Data are means \pm SE, n = 2. * $P < 0.05$ iPFK2-Ctrl vs. iPFK2-KD; [†] $P < 0.05$ Un-treated vs. iPFK2-KD. C, Changes in adipocyte lipid accumulation (arbitrary unit). D, Changes in the rates of glucose incorporation into lipid. Data are means \pm S.E. n = 4. ** $P < 0.01$ iPFK2-KD vs. iPFK2-Ctrl.

PFKFB3/iPFK2 Knockdown Exacerbates Adipocyte Inflammatory Response

Changes in the levels and phosphorylation states of JNK and NF κ B p65 were determined in PFKFB3/iPFK2 knockdown and control adipocytes. Compared to iPFK2-Ctrl adipocytes, iPFK2-KD adipocytes showed an increase in the phosphorylation states of JNK and NF κ B p65 under both basal and LPS-stimulated conditions (Fig. 25 A, B and C). Additionally, the mRNA levels of TNF α and IL-6 were higher in iPFK2-KD adipocytes than in iPFK2-Ctrl adipocytes (Fig. 25 D). In combination, these data indicated that the inflammatory response in iPFK2-KD adipocytes was increased compared to iPFK2-Ctrl adipocytes, although knockdown of PFKFB3/iPFK2 reduced lipid accumulation.

Reactive Oxygen Species (ROS) Generated by Excessive Fatty Acid Oxidation Triggers Adipocyte Inflammatory Response

Since the *in vivo* data suggested a potential role for PFKFB3/iPFK2 in linking fuel metabolism and oxidative stress, the production of ROS in cultured 3T3-L1 cells was determined to further illustrate the underlying mechanisms. Compared to iPFK2-Ctrl adipocytes, iPFK2-KD adipocytes produced much more ROS under both basal and

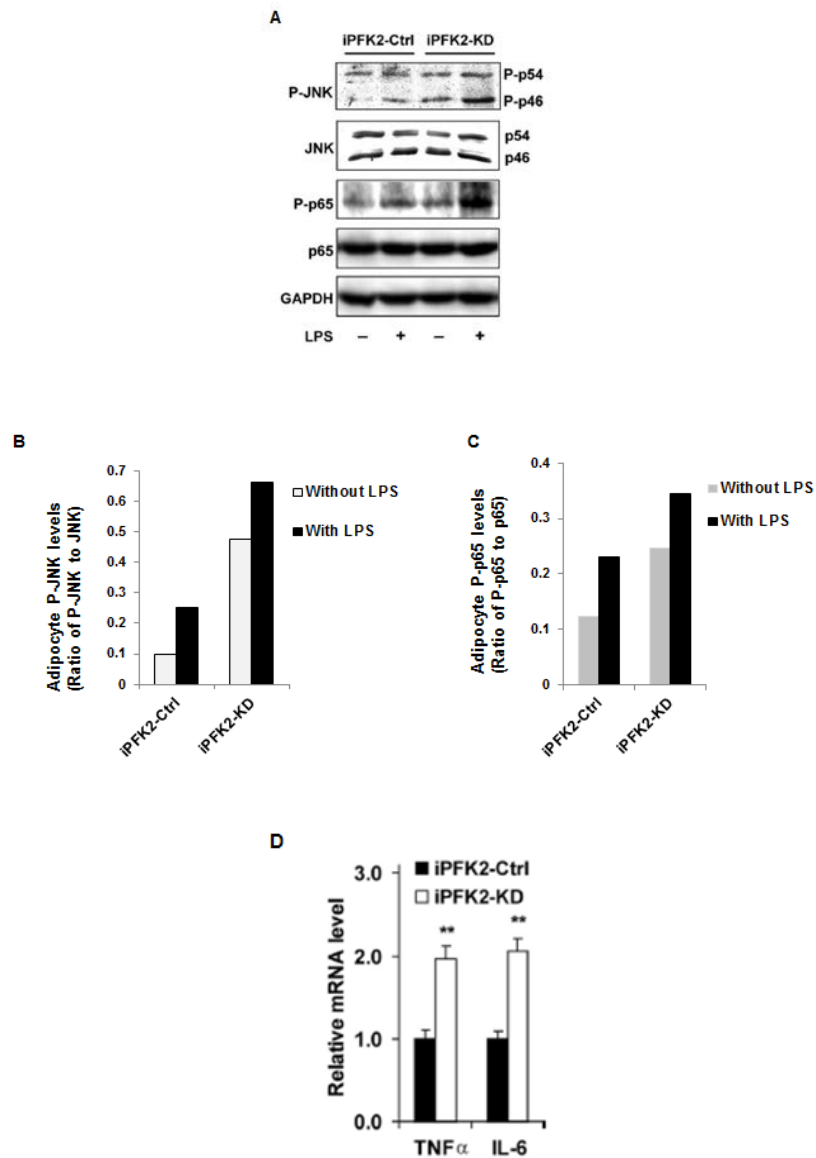


Figure 25. PFKFB3/iPFK2 knockdown exacerbates adipocyte inflammatory response. Predifferentiated 3T3-L1 cells were transfected with the plasmid containing shRNA against PFKFB3/iPFK2 (iPFK2-KD) or shRNA vector (iPFK2-Ctrl) for 24 h and induced for differentiation for 6 – 8 d. Un-transfected cells were differentiated similarly and used as the un-treated control. Some transfected 3T3-L1 cells were selected by puromycin (5 μ g/ml) to establish stable iPFK2-KD or iPFK2-Ctrl cell lines. A, Adipocytes were incubated with or without LPS (100 ng/ml) for 1 h prior to harvest. The levels and phosphorylation states of JNK and NF κ B p65 were determined using Western blots. B and C, Phosphorylation states of JNK and NF κ B p65 were quantified. D, mRNA levels of proinflammatory cytokines were quantified using real-time RT-PCR. Data are means \pm S.E. n = 4. ** $P < 0.01$ iPFK2-KD vs. iPFK2-Ctrl

palmitate-stimulated conditions (Fig. 26 A), indicating an increase in the status of oxidative stress. To determine the contribution of elevated fatty acid oxidation to the increased production of ROS, the effect of etomoxir on ROS production was examined in the stable iPFK2-KD and iPFK2-Ctrl adipocytes. Treatment with etomoxir lowered down the production of ROS in palmitate-stimulated iPFK2-KD adipocytes to the level comparable with those brought about by treatment with N-acetyl-L-cysteine (Nac), a compound that inhibits most general pathways for ROS production (Fig. 26 B). These data suggest that elevated fatty acid oxidation is the major cause of the increased production of ROS when PFKFB3/iPFK2 is disrupted. Oxidative stress, which was formed by excessive ROS, plays a role in triggering inflammatory signaling through JNK and/or NF α B pathways [153, 154].

PFKFB3/iPFK2 Knockdown Induces Adipocyte Dysfunction

Oxidative stress was identified for impairing insulin signaling [153, 154], therefore adipocyte function was determined. The metabolic changes in iPFK2-KD adipocytes were accompanied by an increase in the mRNA levels of resistin and a decrease in the mRNA levels of adiponectin, as well as a decrease in insulin-stimulated phosphorylation of Akt (Fig. 27 A, B and C), indicating impaired adipocyte function.

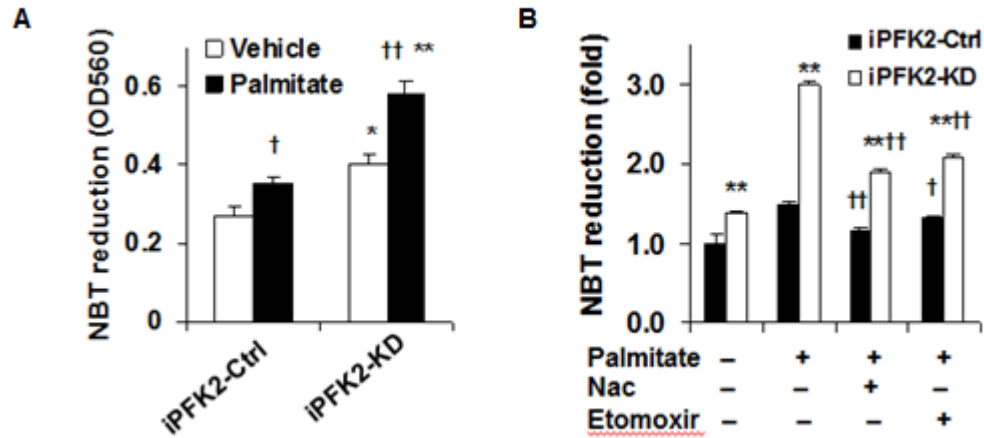


Figure 26. ROS generated by excessive fatty acid oxidation triggers adipocyte inflammatory response. Predifferentiated 3T3-L1 cells were transfected with the plasmid containing shRNA against PFKFB3/iPFK2 (iPFK2-KD) or shRNA vector (iPFK2-Ctrl) for 24 h and induced for differentiation for 6 – 8 d. Un-transfected cells were differentiated similarly and used as the un-treated control. Some transfected 3T3-L1 cells were selected by puromycin (5 μ g/ml) to establish stable iPFK2-KD or iPFK2-Ctrl cell lines. The production of ROS was measured using the NBT assay. A, After differentiation, iPFK2-KD and iPFK2-Ctrl adipocytes were treated with or without palmitate (250 μ M) for 24 h. B, After differentiation, the stable cell lines were treated with or without palmitate (250 μ M) for 24 h. In the last hour of treatment, 100 μ M etomoxir (an inhibitor of carnitine palmitoyltransferase-1, CPT1) or 10 mM N-acetyl-L-cysteine (an antioxidant) was added. Data are means \pm S.E. n = 4. * P < 0.05 and ** P < 0.01 iPFK2-KD vs. iPFK2-Ctrl for the treatment of palmitate or vehicle in A and palmitate or vehicle with or without etomoxir or Nac in B; [†] P < 0.05 and ^{††} P < 0.01 palmitate vs. vehicle in A and palmitate in the presence of etomoxir or Nac vs. palmitate alone in B under the same condition (iPFK2-KD or iPFK2-Ctrl).

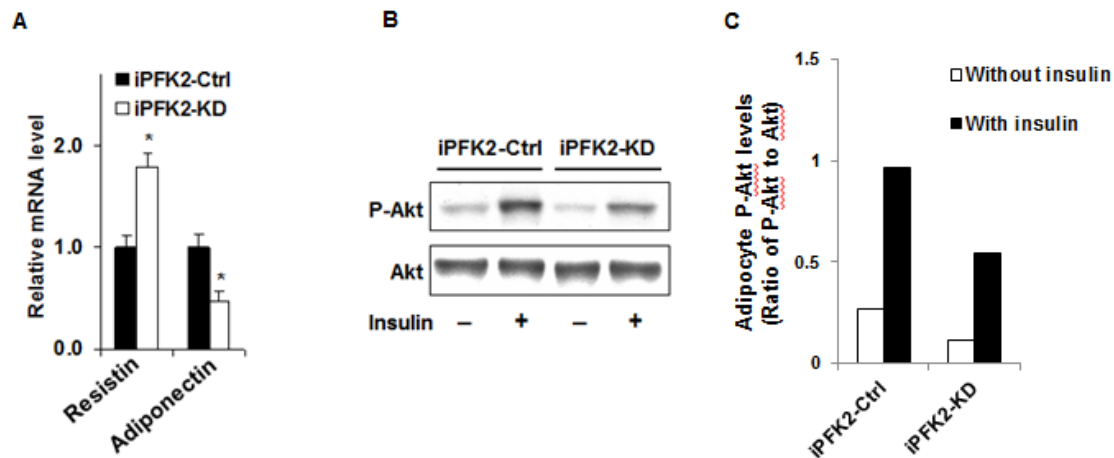


Figure 27. PFKFB3/iPFK2 knockdown induces adipocyte dysfunction. Predifferentiated 3T3-L1 cells were transfected with the plasmid containing shRNA against PFKFB3/iPFK2 (iPFK2-KD) or shRNA vector (iPFK2-Ctrl) for 24 h and induced for differentiation for 6 – 8 d. Un-transfected cells were differentiated similarly and used as the un-treated control. Some transfected 3T3-L1 cells were selected by puromycin (5 µg/ml) to establish stable iPFK2-KD or iPFK2-Ctrl cell lines. A, Changes in the mRNA levels of adipokines were quantified using real-time RT-PCR. B, Adipocyte insulin signaling was analyzed using Western blot. Before harvest, the cells were incubated with or without insulin (100 nM) for 30 min. C, Phosphorylation of Akt and Akt were quantified. Data are means ± S.E. n = 4. * $P < 0.05$ iPFK2-KD vs. iPFK2-Ctrl.

PFKFB3/iPFK2 Overexpression Increases Fat Deposition

Stable adipocytes overexpressing PFKFB3/iPFK2 (iPFK2-OX) were established (Fig. 28 A). Compared with GFP expressing adipocytes (control), iPFK2-OX adipocytes exhibited an increase in rates of glucose incorporation into lipid (Fig. 28 B). The data shows that PFKFB3/iPFK2 overexpression increases fat deposition in adipocytes.

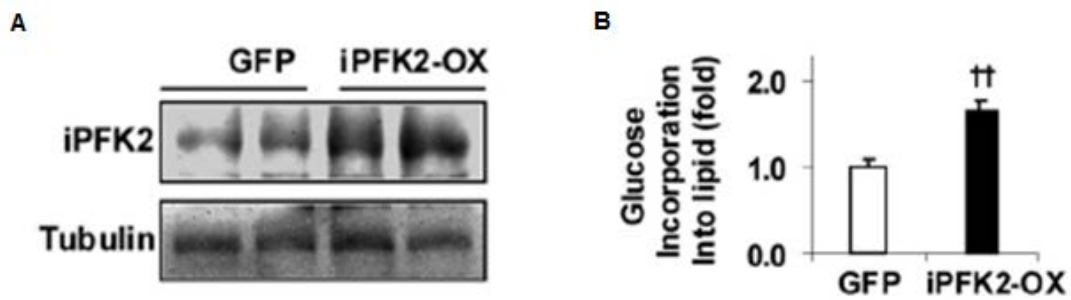


Figure 28. PFKFB3/iPFK2 overexpression increases fat deposition. Stable iPFK2-OX adipocytes and GFP-expressing adipocytes were established and subjected to metabolic and inflammatory assays. Each of the assays was performed at least in quadruplicate. A, amounts of iPFK2 in cells lysates were examined using Western blot analyses. B, changes in the rates of glucose incorporation into lipid. ††, $p < 0.01$ iPFK2-OX vs. GFP.

PFKFB3/iPFK2 Overexpression Dissociates Adipocyte Inflammatory Response and Insulin Resistance from Fat Deposition

iPFK2-OX is used to verify if PFKFB3/iPFK2 plays a direct role for in dissociating the inflammatory response and insulin resistance linked to fat deposition. iPFK2-OX showed a decrease in the generation of reactive oxygen species (ROS) under both basal (BSA) and palmitoleate-stimulated conditions (Fig. 29 A), indicating decreased the status of oxidative stress. Additionally, iPFK2-OX adipocytes showed a decrease in NF- κ B p65 (Ser-468) phosphorylation (Fig. 29 B), as well as IL-6 expression in both basal (PBS) and TNF α -stimulated conditions compared with controls (Fig. 29 C). When insulin signaling was analyzed, iPFK2-OX adipocytes showed an increase in Akt (Ser-473) phosphorylation at both basal and insulin-stimulated conditions compared with control adipocytes (Fig. 29 D). Thus, PFKFB3/iPFK2 has direct effects on decreasing

adipocyte inflammatory response and on improving insulin signaling while increasing fat deposition.

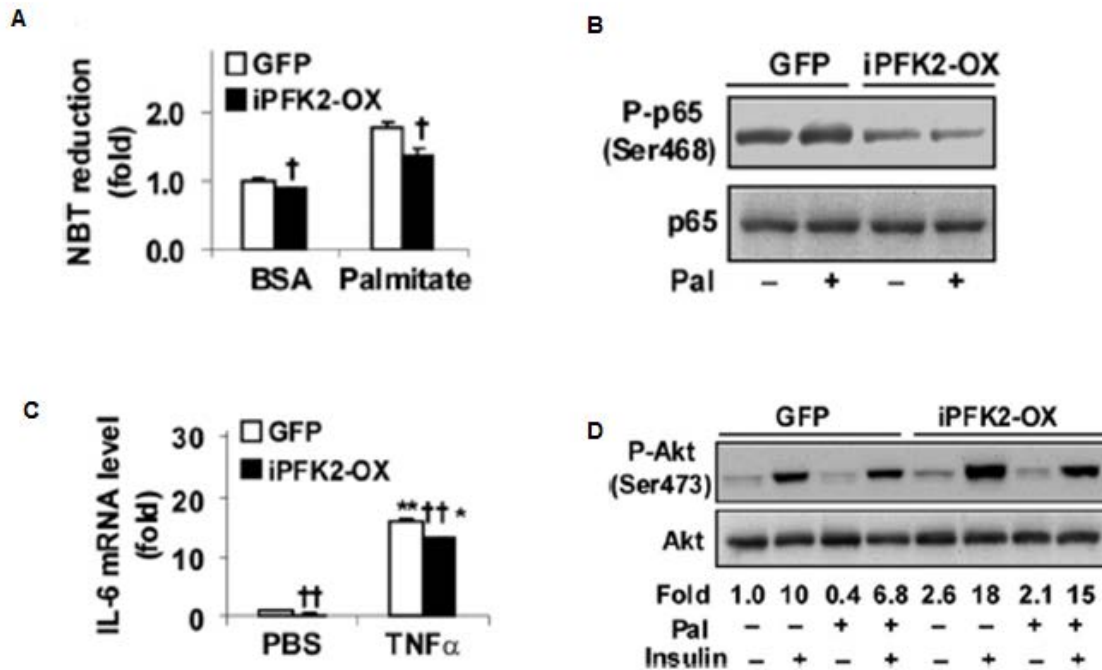


Figure 29. PFKFB3/iPFK2 overexpression decreases adipocyte inflammatory response and improves insulin signaling while increasing fat deposition. Stable iPFK2-OX adipocytes and GFP-expressing adipocytes were established and subjected to metabolic and inflammatory assays. Each of the assays was performed at least in quadruplicate. A, production of ROS was measured using the nitro blue tetrazolium (*NBT*) assay. B, adipocyte inflammatory signaling. The levels of NF- κ B p65 and phospho-p65 (Ser-468) were examined using Western blot analyses. C, adipocyte expression of IL-6. D, adipocyte insulin signaling. Prior to harvest, adipocytes were incubated with or without insulin (100 nM) for 30 min. Phospho-Akt (Ser-473) to Akt1/2 ratios were calculated using densitometry and expressed as fold changes. A-D, adipocytes were incubated with or without palmitate (250 μ M) for 24 h (A, B and D) or TNF α (10 ng/ml) for 6 h (C). A and C, [†], $p < 0.05$; ^{††}, $p < 0.01$ iPFK2-OX vs. GFP under the same condition (A and C). *, $p < 0.05$; **, $p < 0.01$ TNF α vs. PBS.

DISCUSSION

Feeding an HFD to mice induces adiposity, which is associated with adipose tissue dysfunction, a key contributor of systemic insulin resistance [151, 152]. This is the case in wild-type littermates. However, in PFKFB3^{+/-} mice, although bringing about a much smaller increase in adiposity, feeding an HFD caused a much greater increase in the severity of HFD-induced adipose tissue dysfunction and systemic insulin resistance than in wild-type littermates. These changes were attributed, at least in part, to the increased adipose tissue inflammatory response, which was evidenced by higher levels of proinflammatory cytokines in both isolated adipose tissue macrophages and adipocytes in PFKFB3^{+/-} mice. Consistently, in cultured adipocytes, knockdown of PFKFB3/iPFK2 caused a decrease in lipid accumulation and an increase in the status of oxidative stress, which were accompanied by enhanced inflammatory signaling, increased mRNA levels of TNF α and IL-6, and decreased insulin signaling.

As a result of selective PFKFB3/iPFK2 overexpression in adipose tissue, Tg mice exhibited a dissociation of fat deposition, the inflammatory response, and insulin resistance. Locally, adiposity in HFD-fed Tg mice was greater than in wild-type littermates, which is consistent with the role of PFKFB3/iPFK2 in promoting glycolysis and glycolysis-derived lipogenesis as indicated by the results from PFKFB3/iPFK2-overexpressing adipocytes. However, while showing increased adiposity, HFD-fed Tg mice exhibited a decrease in adipose tissue NF- κ B p65 (Ser-468) phosphorylation and proinflammatory cytokine expression while accumulating more macrophages. Because

aP2-PFKFB3 primarily elevated PFKFB3/iPFK2, it is postulated that PFKFB3/iPFK2 is capable of mediating a dissociation of fat deposition and the inflammatory response. In support of this finding, PFKFB3/iPFK2 overexpression in adipocytes increased fat accumulation but decreased adipocyte inflammatory response. In PFKFB3/iPFK2 overexpressing adipocytes, adipocyte ROS production and the inflammatory response are reduced while increasing fat accumulation. By altering lipid composition, PFKFB3/iPFK2 may dissociate adipose tissue/adipocyte fat deposition and the inflammatory response. When the inflammatory response was decreased, insulin signaling through Akt was improved in both adipose tissue of Tg mice and in PFKFB3/iPFK2-overexpressing adipocytes.

Together, these data argue in favor of a novel and unique role for PFKFB3/iPFK2 in regulating HFD-induced adipose tissue dysfunction and systemic insulin resistance in a manner independent of adiposity.

In summary, the present study demonstrates a novel and unique role for PFKFB3/iPFK2 in regulating adiposity and adipose tissue function, and thereby systemic insulin sensitivity. This role is manifested by the fact that disruption of PFKFB3/iPFK2 ameliorates HFD-induced adiposity, but exacerbates HFD-induced adipose tissue dysfunction, in particular, adipose tissue inflammatory response, which contributes to an increase in the severity of systemic insulin resistance. Additionally, adipocyte-specific PFKFB3 over-expression increased adiposity but suppressed overnutrition induced adipose tissue inflammatory response and improved insulin

sensitivity. Thus, PFKFB3/iPFK2 protects against HFD-induced inflammation and insulin resistance independent of adiposity.

CHAPTER IV

THE ROLE OF PFKFB3/iPFK2 IN THE ANTI-DIABETIC EFFECT OF PPAR γ ACTIVATION*

INTRODUCTION

Peroxisome proliferator-activated receptor γ (PPAR γ) is a nuclear receptor whose activation by thiazolidinediones (TZDs) effectively improves systemic insulin sensitivity and lowers plasma glucose levels in both human patients and rodent models of type 2 diabetes [76-82, 155]. Because TZDs are effective in liver- or muscle-specific PPAR γ - deficient mice [87, 88] but not in adipose tissue-deleted mice [89] and adipose-specific PPAR γ -deficient mice [90], adipose tissue has been considered as the primary target site for the anti-diabetic effect of PPAR γ activation [75, 91]. Further investigations have suggested two adipose tissue-based mechanisms to largely explain the anti-diabetic effect of PPAR γ activation [45, 81]. In the first mechanism, PPAR γ activation by TZDs appropriately alters the expression of adipocyte genes that are involved in lipogenesis and triglyceride synthesis to increase the capacity of fat storage in adipose tissue [58, 94-96, 100]. This leads to reduction of the circulating levels of free fatty acids (FFA) and thereby reversal of FFA-induced insulin resistance. In the second mechanism, PPAR γ

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activation by TZDs suppresses the adipose tissue inflammatory response [58, 97] and appropriately regulates adipokine expression [81, 98, 99]. This improves adipose tissue function, which in turn brings about the insulin-sensitizing effect [81]. However, the molecular link between the two mechanisms remains to be elucidated.

Many PPAR γ target genes are changed to promote fat storage in adipose tissue [100-103] when PPAR γ is activated. TZDs stimulate the expression of Glycerol Kinase (GyK), which increases triglyceride synthesis in adipocytes by providing glycerol 3-phosphate as a key substrate [100]. The activation of PPAR γ also stimulates the expression of the cyto-solic isoform of Phosphoenolpyruvate Carboxykinase (PEPCK) in adipocytes [101, 102]. This increases glyceroneogenesis and provides another way to synthesize triglycerides in adipocytes/adipose tissue [104, 105]. In response to TZDs, the expression of several proinflammatory genes is decreased in the adipose tissue in both rodents and human patients with type 2 diabetes [95, 108]. Further, PPAR γ activation in both adipose tissue macrophages and adipocytes contributes to the suppression of the adipose tissue inflammatory response [97, 109]. Currently, mediators that are involved in the effect of PPAR γ activation on adipocyte inflammatory response are largely unknown.

PFKFB3/iPFK2 is a target gene of PPAR γ [156] and is stimulated by TZDs [110]. The expression of PFKFB3/iPFK2 is at high levels in adipose tissue and at very low levels in the liver and skeletal muscle [110, 133]. PFKFB3/iPFK2 plays a pivotal role for in regulating adipose tissue function and systemic insulin sensitivity in our recent study. Mechanistically, PFKFB3/ iPFK2 protects against fatty acid oxidation-

associated reactive oxygen species (ROS) production, thereby reducing inflammatory signaling through JNK1 and NF- κ B pathways to suppress adipocyte inflammatory response. However, the extent to which PFKFB3/iPFK2 participates in the *in vivo* effects of PPAR γ activation is not known. Using PFKFB3/iPFK2-disrupted mice, the present study found that PFKFB3/iPFK2 is involved in the anti-diabetic effect of PPAR γ activation, likely by increasing the ability of adipose tissue to store fat and by suppressing adipose tissue inflammatory response. In addition, the mechanisms underlying the involvement of PFKFB3/iPFK2 in the effects of PPAR γ activation are explored in cultured 3T3-L1 adipocytes.

MATERIALS AND METHODS

Animal Experiments

Because homozygous disruption of PFKFB3/iPFK2 is embryonic lethal [133], PFKFB3^{+/-} mice were generated as described previously [133] and used for the present study. Considering that rosiglitazone lowers the levels of plasma glucose and improves systemic insulin sensitivity only in diabetic mice, male PFKFB3^{+/-} and wildtype littermates (C57BL/6J background) were fed a high fat diet (HFD) prior to treatment with rosiglitazone. Feeding an HFD to PFKFB3^{+/-} mice exacerbates systemic insulin resistance and adipose tissue inflammatory response. Briefly, all mice were maintained as previously described. At the age of 5–6 weeks, mice were fed an HFD (60% fat calories, 20% protein calories, and 20 carbohydrate calories) (Research Diets, Inc., New

Brunswick, NJ) for 12 weeks. During the last 4 weeks of the feeding regimen, HFD-fed mice were treated with rosiglitazone (10 mg/kg/day in PBS; Avandia tablets) or vehicle (PBS) via oral gavages. As the control, the age-matched male mice were fed a low fat diet (10% fat calories, 20% protein calories, and 70% carbohydrate calories) and received no treatment. The composition of both HFD and low fat diet has been described previously. Body weight and food intake of the mice were recorded every 4 days during the 12-week feeding period. At the end of the feeding/treatment regimen, mice were fasted for 4 h before sacrifice for collection of blood and tissue samples as described previously [135-137]. Visceral fat content was estimated as the sum of epididymal, mesenteric, and perinephric fat depots [136]. After weighing, lipolysis rates were determined on adipose tissue samples. Some tissue samples were either fixed and embedded for histological analyses or frozen in liquid nitrogen and then stored at - 80 °C for further analyses. Some mice were fasted similarly and used for glucose and insulin tolerance tests. For a separate study to analyze the role of PPAR γ in regulating PFKFB3 expression, PPAR γ 2^{+/-} mice and their wild-type littermates were generated as described before [120] and used for adipose tissue sample collection and embryonic fibroblast isolation. All study protocols were reviewed and approved by the Institutional Animal Care and Use Committee of Texas A&M University.

Measurement of Metabolite and Hormone Levels

The levels of plasma metabolites, such as glucose, triglycerides, and FFA, were measured using metabolic assay kits (Sigma and BioVision (Mountain View, CA)). The

levels of plasma hormones (*i.e.* insulin and leptin) were measured using enzyme-linked immunosorbent assay kits (Crystal Chem Inc., Downers Grove, IL). The levels of adipose tissue fructose 2,6-bisphosphate (F26P2) were determined using the 6-phosphofructo-1-kinase activation method as described previously [137].

Glucose and Insulin Tolerance Tests

The assays were conducted as previously described [137]. After fasting for 4 h, mice received a peritoneal injection of D-glucose (2 g/kg) or insulin (0.5 unit/kg) (Humulin, Lily).

Measurement of Adipose Tissue Lipolysis

The assays were conducted as described previously [138, 139]. Briefly, freshly isolated adipose tissue samples were washed several times with PBS and incubated in a final volume of 1 ml of high glucose Dulbecco's modified Eagle's medium containing 2% fatty acid free bovine serum albumin in the presence or absence of 10 μ M isoproterenol at 37 °C for 3 h. Aliquots of the medium were sampled hourly to quantify glycerol content using metabolic kits (BioVision, Mountain View, CA). The rate of lipolysis was estimated as the efflux of glycerol.

Histological Analyses of Adipose Tissue

The paraffin-embedded adipose tissue blocks were cut into sections of 5- μ m thickness and stained with hematoxylin and eosin.

Cell Culture and Treatment

3T3-L1 cells were maintained in high glucose Dulbecco's modified Eagle's medium and differentiated in induction medium for 6–8 days as described previously. To explore the direct role of PFKFB3/iPFK2 in modulating the effect of PPAR γ activation, stable PFKFB3/iPFK2-knockdown (iPFK2-KD) 3T3-L1 cells and control (iPFK2-Ctrl) 3T3-L1 cells were used. These cells have been previously established. During the last 48 h of differentiation, both iPFK2-KD and iPFK2-Ctrl adipocytes were treated with rosiglitazone (1 μ M) or vehicle (0.1% DMSO) and used to quantify the expression of PPAR γ target genes as well as for further analyses using the methods described below.

To quantify adipocyte lipid content, the cells were stained with Oil Red O. The lipid-associated dye was extracted with isopropyl alcohol for 15 min. The OD of the extraction solution was measured using a spectrophotometer at 510 nm [120]. To determine the rate of glucose incorporation into lipid, each well (6-well plate) of the cells was incubated with Dulbecco's modified Eagle's medium supplemented with 1 μ Ci of [U- 14 C] glucose for 24 h as described previously [141]. After sequential extraction with 30% KOH, 95% ethanol, 9 M H₂SO₄, and petroleum ether, the amount of 14 C-labeled lipids was quantified using a Beckman liquid scintillation counter. To analyze inflammatory signaling, the cells were incubated with palmitate (250 μ M) or vehicle (0.5% bovine serum albumin) for 24 h. Cell lysates were then prepared and used for

Western blots to measure the levels and phosphorylation states of JNK and NF- κ B p65. To determine adipocyte expression of proinflammatory cytokines and adipokines, the total RNA of the cells was prepared and used for real-time RT-PCR. To determine changes in insulin signaling, the cells were treated with or without insulin (100 nM) for 30 min prior to harvest. Cell lysates were prepared and used to measure the levels and phosphorylation of Akt using Western blots.

PFKFB3/iPFK2-knockdown-associated increase in fatty acid oxidation triggers adipocyte inflammatory response, which may account for lessening or blunting of the anti-inflammatory effect of PPAR γ activation. To verify this concept, the differentiated stable iPFK2-KD and iPFK2-Ctrl adipocytes were treated with rosiglitazone (1 μ M) or vehicle (0.1% DMSO) for 48 h. In the last 24 h, 100 μ M etomoxir (an inhibitor of carnitine palmitoyltransferase-1), with or without palmitate (250 μ M), was added to rosiglitazone-treated cells. Thereafter, the cells were used to measure the production of ROS using the nitro blue tetrazolium assay as described previously [142] or harvested to analyze adipocyte inflammatory response and insulin signaling as described above.

RNA Isolation, Reverse Transcription, and Real-time PCR

The total RNA was isolated from frozen tissue samples and cultured cells. RNA isolation and real-time RT-PCR were conducted as described previously [137]. The mRNA levels were analyzed for PFKFB3, GlyK, PEPCK, PPAR γ , resistin, adiponectin, tumor necrosis factor α (TNF α), and interleukin-6 (IL-6) in adipose tissue samples and/or cell samples.

Western Blots

Lysates were prepared from frozen tissue samples and cultured cells. Western blots were conducted as described previously [136, 137]. The levels of JNK, phospho-JNK, NF- κ B p65, phospho-p65, Akt1/2, and phospho-Akt (Ser473) were analyzed.

Statistical Methods

Numeric data are presented as means \pm S.E. Statistical significance was assessed by unpaired, two-tailed analysis of variance or Student's *t* test. Differences were considered significant at the two-tailed $p < 0.05$.

RESULTS

Disruption of PFKFB3/iPFK2 Blunts the Anti-diabetic Effect of PPAR γ Activation

After a feeding of an HFD, wild-type littermates exhibited insulin resistance, glucose intolerance, and hyperglycemia. In these wild-type mice, treatment with rosiglitazone for 4 weeks normalized the levels of plasma glucose (169.67 ± 6.05 *versus* 242.59 ± 6.23 mg/dl, $p < 0.01$; Fig. 30 A) and corrected glucose intolerance and insulin resistance (Fig. 30, B and C) compared with vehicle. Additionally, treatment with rosiglitazone lowered the circulating levels of insulin (Fig. 30 D), indicating the reversal of hyperinsulinemia. These data confirmed the anti-diabetic effect of PPAR γ activation. Consistent with our previous study, feeding an HFD to PFKFB3^{+/-} mice exacerbated systemic insulin resistance and adipose tissue inflammatory response. In these

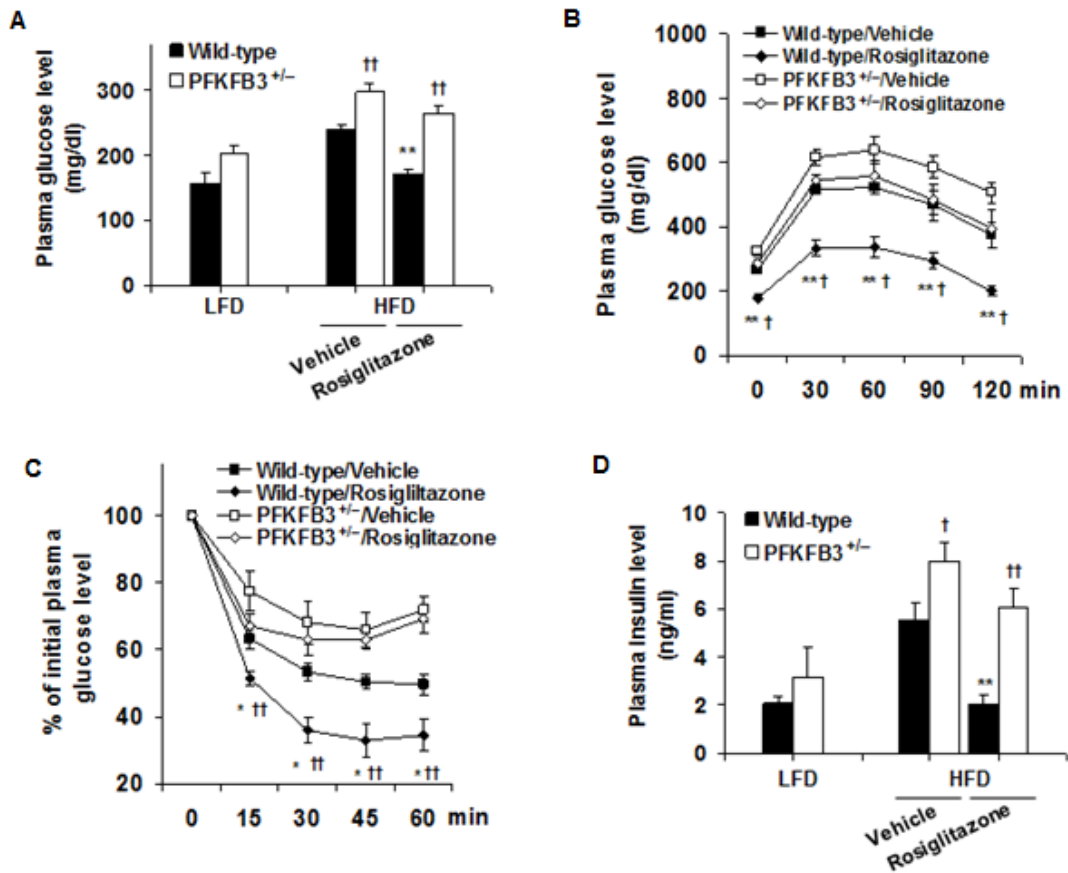


Figure 30. Disruption of PFKFB3/iPFK2 blunts the anti-diabetic effect of PPAR γ activation. Male PFKFB3^{+/-} mice and wild-type littermates, at the age of 5–6 weeks, were fed an HFD for 12 weeks and treated with rosiglitazone (10 mg/kg/day) or vehicle (PBS) during the last 4 weeks of HFD feeding. Data are means \pm S.E. (error bars), $n = 6$. A, changes in the levels of plasma glucose. As the control, the age-matched male PFKFB3^{+/-} mice and wild-type littermates were fed a low fat diet (LFD) and received no treatment. All of the mice were fasted for 4 h before collection of blood samples. **, $p < 0.01$, rosiglitazone versus vehicle within the same genotype. †, $p < 0.05$; ††, $p < 0.01$, PFKFB3^{+/-} vs. wild type on an HFD with the same treatment (rosiglitazone or vehicle). For B and C, mice were fasted for 4 h and received an intraperitoneal injection of D-glucose (2 g/kg) (B) or insulin (0.5 units/kg) (C). *, $p < 0.05$; **, $p < 0.01$, wild type/rosiglitazone vs. wild type/vehicle. †, $p < 0.05$; ††, $p < 0.01$, PFKFB3^{+/-}/rosiglitazone vs. wild type/rosiglitazone. B, glucose tolerance test. C, insulin tolerance test. D, changes in the levels of plasma insulin. Mice were fed and/or treated as described in A. Statistical analyses were identical to those in A.

PFKFB3/iPFK2- disrupted mice, treatment with rosiglitazone only caused an insignificant decrease in the levels of plasma glucose (263.14 ± 13.20 *versus* 297.05 ± 12.30 mg/dl; Fig. 30 A) and failed to improve insulin resistance and glucose intolerance. Thus, disruption of PFKFB3/iPFK2 blunts the anti-diabetic effect of PPAR γ activation.

Disruption of PFKFB3/iPFK2 Impairs the Response of Adipose Tissue PFKFB3/iPFK2 to PPAR γ Activation

TZDs primarily target adipose tissue [89, 90] and stimulate the expression of PFKFB3/iPFK2 in adipocytes [110]. To address the link between adipose tissue PFKFB3/iPFK2 and the anti-diabetic effect of PPAR γ activation, the response of adipose tissue PFKFB3/iPFK2 to rosiglitazone was determined. Compared with vehicle, rosiglitazone treatment caused a significant increase in the mRNA levels of adipose PFKFB3/iPFK2 in wild-type littermates, confirming the stimulatory effect of PPAR γ activation. However, this stimulatory effect was markedly lessened in PFKFB3^{+/-} mice (Fig. 31, A and B). Because PFKFB3/iPFK2 determines the production of F26P2, the levels of adipose tissue F26P2 were quantified to reflect PFKFB3/iPFK2 activity. Consistent with an increase in the mRNA levels of PFKFB3/iPFK2, the levels of adipose tissue F26P2 were significantly higher in rosiglitazone-treated wild-type mice than in vehicle-treated wild-type mice (3.54 ± 0.48 *versus* 1.77 ± 0.21 nmol/g, $p < 0.05$; Fig. 31 C). However, in PFKFB3^{+/-} mice, rosiglitazone only caused a slight and insignificant increase in the levels of F26P2 compared with vehicle (1.29 ± 0.29 *versus* 0.92 ± 0.08

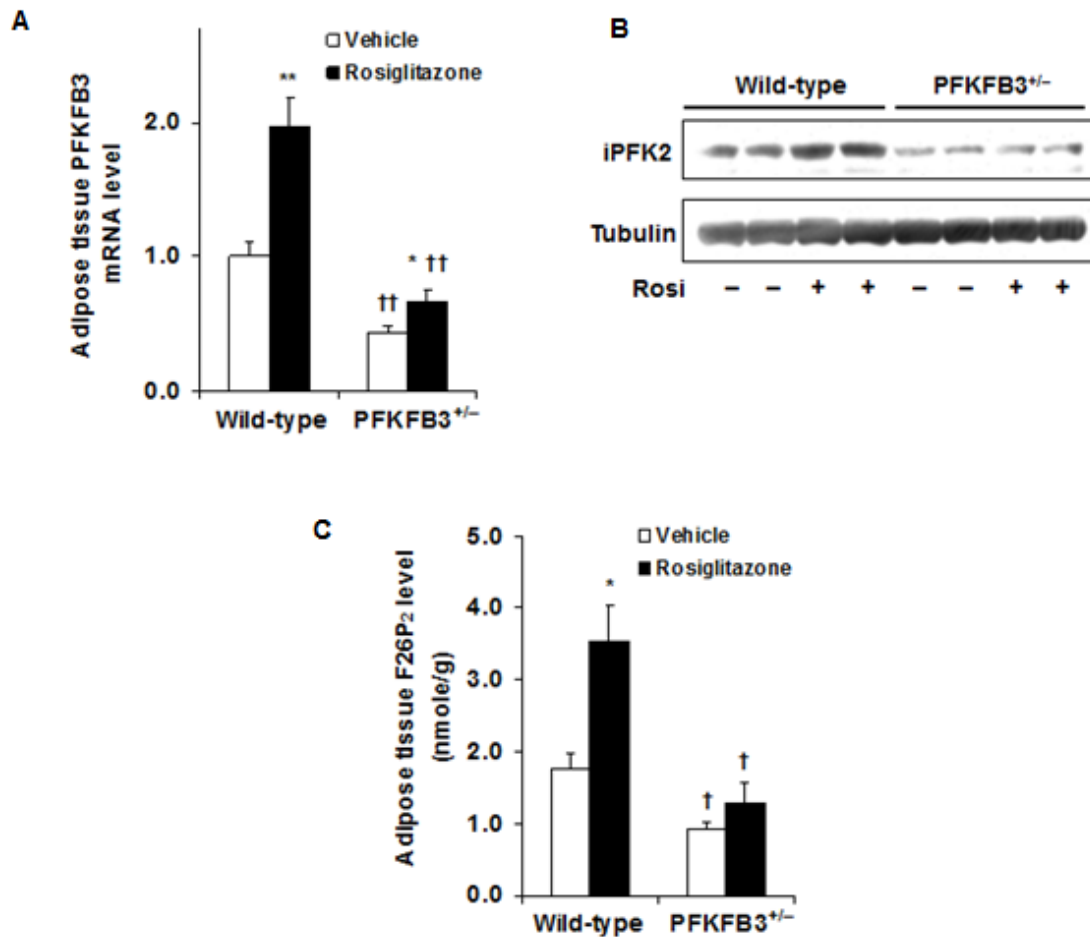


Figure 31. Disruption of PFKFB3/iPFK2 impairs the response of adipose tissue PFKFB3/iPFK2 to PPAR γ activation. At the age of 5– 6 weeks, male PFKFB3^{+/-} mice and wild-type littermates were fed an HFD for 12 weeks and treated with rosiglitazone (10 mg/kg/day) or vehicle (PBS) during the last 4 weeks of HFD feeding. At the end of the feeding/treatment regimen, mice were fasted for 4 h before collection of tissue samples. Epididymal adipose tissue samples were used for the analyses. A, the mRNA levels of PFKFB3 were measured using real-time RT-PCR. B, adipose tissue iPFK2 was determined using Western blot. C, adipose tissue F26P2 levels were determined using the 6-phosphofructo-1-kinase activation method. For A and C, data are means \pm S.E. (error bars), n=6. *, p < 0.05; **, p < 0.01, rosiglitazone vs. vehicle within the same genotype (in A and C). †, p < 0.05; ††, p < 0.01, PFKFB3^{+/-} vs. wild type with the same treatment (rosiglitazone or vehicle in A and C).

nmol/g). These data suggest that two intact PFKFB3/iPFK2 alleles are necessary for mice to fully respond to PPAR γ activation.

Disruption of PFKFB3/iPFK2 Does not Impair the Response of Other PPAR γ Target Genes to PPAR γ Activation

To address whether disruption of PFKFB3/iPFK2 impairs the response of other PPAR γ target genes to PPAR γ activation, the expression of GyK and PEPCK as well as PPAR γ in the adipose tissue was determined. Under the basal condition (treatment with vehicle), the expression of the GyK, PEPCK, and PPAR γ in PFKFB3^{+/-} mice did not differ from that in wild-type mice (Fig. 32, A and B). Furthermore, in response to PPAR γ activation by rosiglitazone treatment, the expression of GyK and PEPCK in PFKFB3^{+/-} mice was increased to an extent comparable with that in wild-type littermates (Fig. 32, A and B). These data indicate that PPAR γ expression, PPAR γ translocation to cell nuclei, and activation of PPAR γ -targeted genes are not disturbed in PFKFB3^{+/-} mice.

Disruption of PFKFB3/iPFK2 Lessens the Effect of PPAR γ Activation on Increasing Adipose Tissue Fat Storage

Increasing adipose tissue fat storage, to a large extent, accounts for the anti-diabetic and insulin-sensitizing effects of PPAR γ activation [95, 100, 103]. Changes in the rate of adipose tissue lipolysis, the content of visceral fat, and the size of epididymal

adipocytes were determined to address the extent to which PPAR γ activation increases the ability of adipose tissue to store fat in the mice. In wild-type littermates, treatment

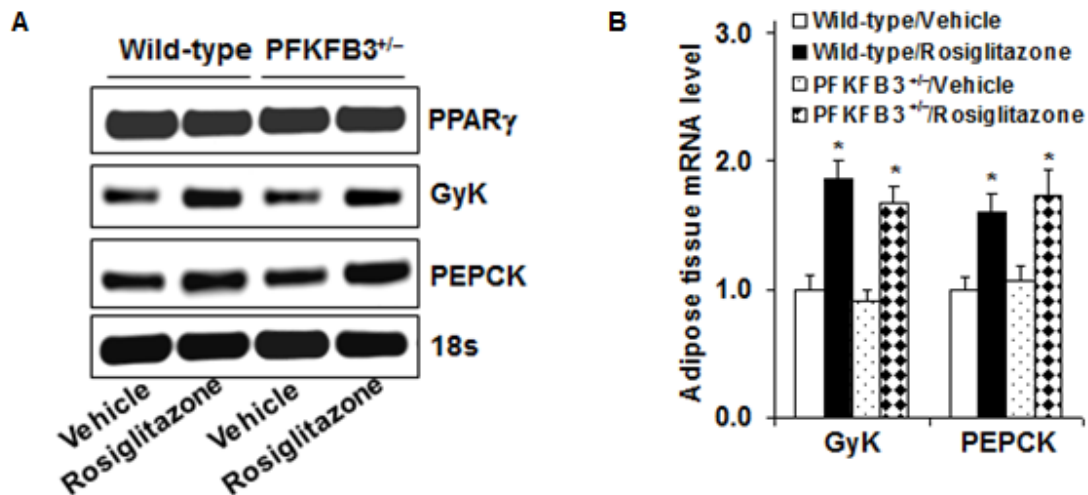


Figure 32. Disruption of PFKFB3/iPFK2 does not impair the response of other PPAR γ target genes to PPAR γ activation. At the age of 5– 6 weeks, male PFKFB3^{+/-} mice and wild-type littermates were fed an HFD for 12 weeks and treated with rosiglitazone (10 mg/kg/day) or vehicle (PBS) during the last 4 weeks of HFD feeding. At the end of the feeding/treatment regimen, mice were fasted for 4 h before collection of tissue samples. Epididymal adipose tissue samples were used for the analyses. A, representative PCR products of adipose tissue genes. B, quantification of the expression of adipose tissue genes. Rosi, rosiglitazone. For E, data are means \pm S.E.(error bars), n=6. *, p < 0.05; **, p < 0.01, wild type/rosiglitazone vs. wild type/vehicle and PFKFB3^{+/-}/rosiglitazone vs. PFKFB3^{+/-}/vehicle for the same gene.

with rosiglitazone caused a significant decrease in adipose tissue lipolysis and an increase in visceral fat content (Fig. 33, A and B). However, in PFKFB3^{+/-} mice, treatment with rosiglitazone did not bring about significant changes in any of the above

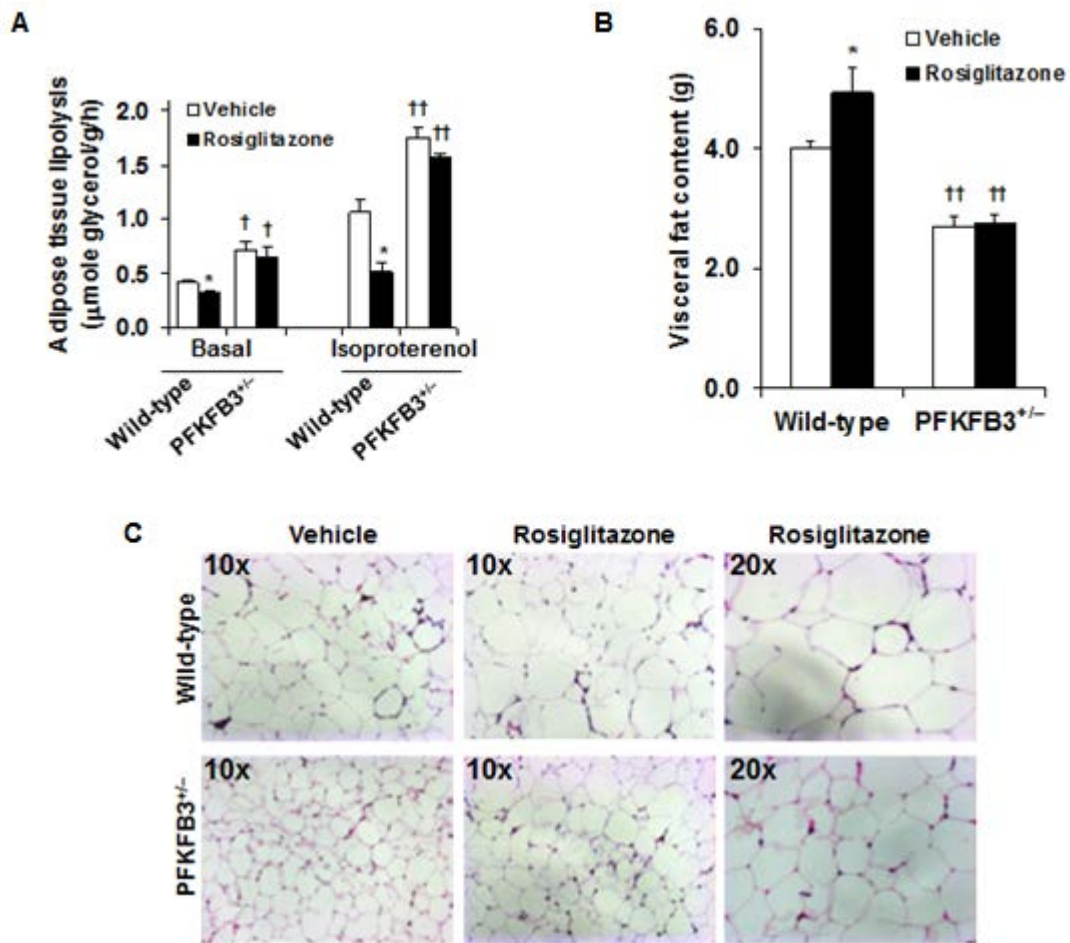


Figure 33. Disruption of PFKFB3/iPFK2 lessens the effect of PPAR γ activation on increasing adipose tissue fat storage. At the age of 5–6 weeks, male PFKFB3^{+/-} mice and wild-type littermates were fed anHFD for 12 weeks and treated with rosiglitazone (10 mg/kg/day) or vehicle (PBS) during the last 4 weeks of HFD feeding. At the end of the feeding/treatment regimen, mice were fasted for 4 h before collection of tissue samples. For A and B, data are means \pm S.E. (error bars), $n = 6$. *, $p < 0.05$ rosiglitazone vs. vehicle within the same genotype (in A and B) in the presence of the same condition (in A, basal or isoproterenol). †, $p < 0.05$; ††, $p < 0.01$, PFKFB3^{+/-} vs. wild type with the same treatment (in A and B, rosiglitazone or vehicle) in the presence of the same condition (in A, basal or isoproterenol). A, the rates of adipose tissue lipolysis were measured under both basal and isoproterenol-stimulated conditions. B, visceral fat content was estimated from the sum of epididymal, mesenteric, and perinephric fat mass. C, adipose tissue histology. The sections of epididymal fat pad were stained with hematoxylin and eosin.

parameters. Additionally, the size of epididymal adipocytes was much larger in rosiglitazone-treated wild-type mice than in rosiglitazone-treated PFKFB3^{+/-} mice (Fig. 33 C). Together, these data demonstrate that disruption of PFKFB3/iPFK2 lessens the effect of PPAR γ activation on increasing adipose tissue fat storage.

Disruption of PFKFB3/iPFK2 Blunts the Effects of PPAR γ Activation on Suppression of HFD-induced Adipose Tissue Inflammatory Response and on Reversal of Adipose Tissue Dysfunction

Suppression of adipose tissue inflammatory response is one of the major mechanisms by which PPAR γ activation reverses insulin resistance and corrects hyperglycemia [81, 97, 157]. The effects of PPAR γ activation on adipose tissue inflammatory signaling and proinflammatory cytokine expression were determined. In HFD-fed wild-type littermates, treatment with rosiglitazone brought about a decrease in the phosphorylation of JNK1 and NF- κ B p65 (Fig. 34, A and B), which was accompanied by a significant decrease in the mRNA levels of TNF α and IL-6 compared with treatment with vehicle (Fig. 34 C). Because suppression of adipose tissue inflammatory response is linked to reversal of adipose tissue dysfunction [81, 158], adipose expression of adipokines and insulin signaling were analyzed. Compared with vehicle, treatment with rosiglitazone caused a decrease in adipose mRNA levels of resistin and an increase in adipose mRNA levels of adiponectin (Fig. 34 D) and an increase in insulin-stimulated phosphorylation of Akt (Fig. 34 E), all of which contributed to the effects of PPAR γ activation on reversal of systemic insulin resistance

and on correction of hyperglycemia. However, in PFKFB3^{+/-} mice, treatment with rosiglitazone did not effectively suppress adipose tissue inflammatory signaling and the mRNA levels of TNF α and IL-6 compared with treatment with vehicle (Fig. 34, A–C). Additionally, treatment with rosiglitazone did not appropriately alter adipose expression of resistin and adiponectin as it did in wild-type mice (Fig. 34 D) and failed to increase insulin-stimulated phosphorylation of Akt (Fig. 34 E). Collectively, these data demonstrate that disruption of PFKFB3/iPFK2 blunts the effects of PPAR γ activation on suppressing HFD-induced adipose inflammatory response and on reversing adipose tissue dysfunction.

Knockdown of PFKFB3/iPFK2 Lessens the Effect of PPAR γ Activation on Stimulating Adipocyte Lipid Accumulation

The direct role of PFKFB3/iPFK2 in modulating the effect of PPAR γ activation on lipid accumulation was explored in iPFK2-KD and iPFK2-Ctrl 3T3-L1 adipocytes. Compared with that in iPFK2-Ctrl cells, the amount of iPFK2 was low and was not increased by rosiglitazone treatment in iPFK2-KD adipocytes (Fig. 35 A). Under the basal condition (treatment with vehicle), iPFK2-KD adipocytes accumulated less lipid than did iPFK2- Ctrl adipocytes (Fig. 35, B and C), which was attributed to a decrease in the rate of glucose incorporation into lipid (Fig. 35 D). After treatment with rosiglitazone, iPFK2-KD adipocytes exhibited a much smaller increase in lipid accumulation and the rate of glucose incorporation into lipid than did iPFK2-Ctrl

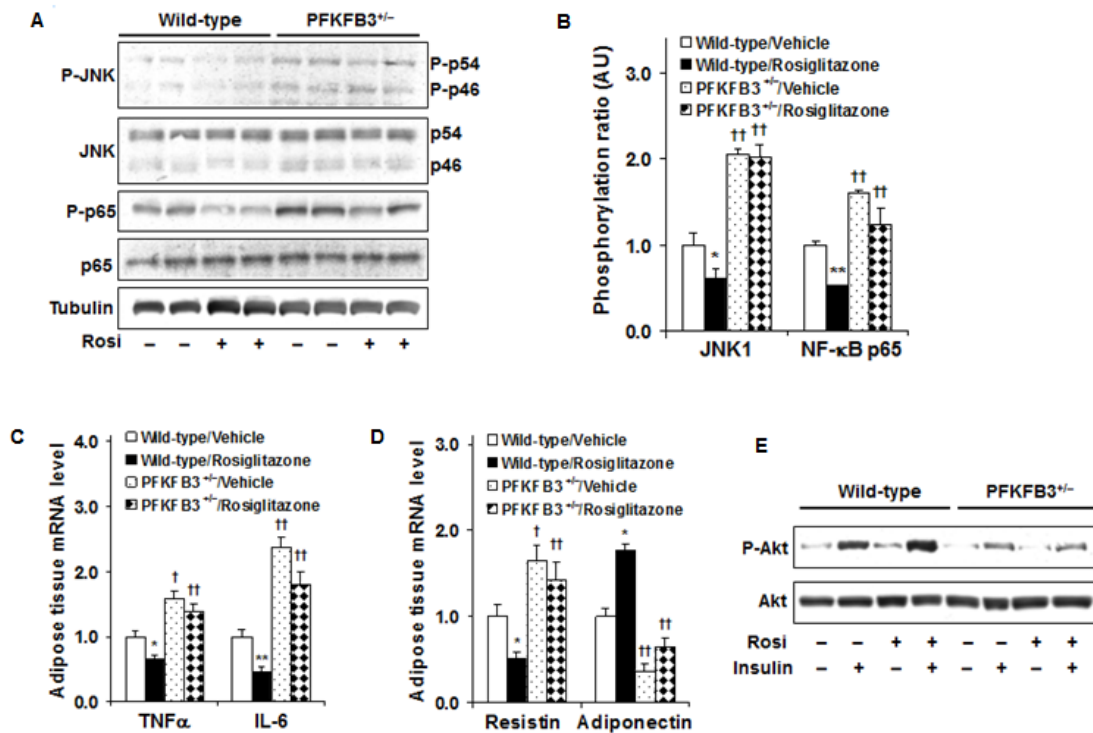


Figure 34. Disruption of PFKFB3/iPFK2 blunts the effects of PPAR γ activation on suppression of HFD induced adipose tissue inflammatory response and on reversal of adipose tissue dysfunction. At the age of 5–6 weeks, male PFKFB3^{+/-} mice and wild-type littermates were fed an HFD for 12 weeks and treated with rosiglitazone (10 mg/kg/day) or vehicle (PBS) during the last 4 weeks of HFD feeding. At the end of the feeding/treatment regimen, mice were fasted for 4 h before the collection of tissue samples. Rosi, rosiglitazone. A, changes in inflammatory signaling were analyzed using Western blots. B, quantification of inflammatory signaling (arbitrary units). C, changes in adipose mRNA levels of TNF α and IL-6. D, changes in adipose mRNA levels of resistin and adiponectin. For B and D, data are means \pm S.E. (error bars), $n = 6$. *, $p < 0.05$; **, $p < 0.01$, wild type/rosiglitazone versus wild type/vehicle for the same gene. †, $p < 0.05$; ††, $p < 0.01$ PFKFB3^{+/-}/vehicle versus wild type/vehicle or PFKFB3^{+/-}/rosiglitazone versus wild type/rosiglitazone for the same gene. For C and D, the expression of adipose tissue genes was measured using real-time RT-PCR. E, adipose tissue insulin signaling was analyzed using Western blot. Adipose tissue samples were collected at 5 min after a bolus injection of insulin (1 unit/kg) into the portal vein. P-p54, phospho-p54; P-p46, phospho-p46; P-Akt, phospho-Akt.

adipocytes. This occurred where the response of GyK and PEPCK to PPAR γ activation in iPFK2-KD adipocytes was comparable with that in iPFK2-Ctrl adipocytes (data not shown), indicating the importance of PFKFB3/iPFK2 to the effect of PPAR γ activation on stimulating adipocyte lipid accumulation. These data, consistent with those observed in rosiglitazone- treated PFKFB3^{+/-} mice, demonstrate that adipocyte PFKFB3/iPFK2 is needed, at least in part, for PPAR γ activation to increase adipose tissue fat storage.

Knockdown of PFKFB3/iPFK2 Diminishes the Effects of PPAR γ Activation on Suppression of Adipocyte Inflammatory Response

The direct role of PFKFB3/iPFK2 in modulating the effects of PPAR γ activation on adipocyte inflammatory response and on adipocyte function was explored. In iPFK2-Ctrl adipocytes, treatment with rosiglitazone caused a significant decrease in palmitate-stimulated phosphorylation of JNK1 and NF- κ B p65 (Fig. 36, A and B), which was accompanied by a decrease in the mRNA levels of TNF α and IL-6 (Fig. 36 C). In contrast, treatment of iPFK2-KD adipocytes with rosiglitazone did not effectively bring about a significant decrease in palmitate-stimulated phosphorylation of JNK1 and NF- κ B p65 and failed to suppress the mRNA levels of TNF α and IL-6 (Fig. 36, A–C). These data, along with those observed in rosiglitazone-treated PFKFB3^{+/-} mice, suggest that the intact PFKFB3/iPFK2 in adipocytes is needed, at least in part, for PPAR γ activation to suppress adipose tissue inflammatory response.

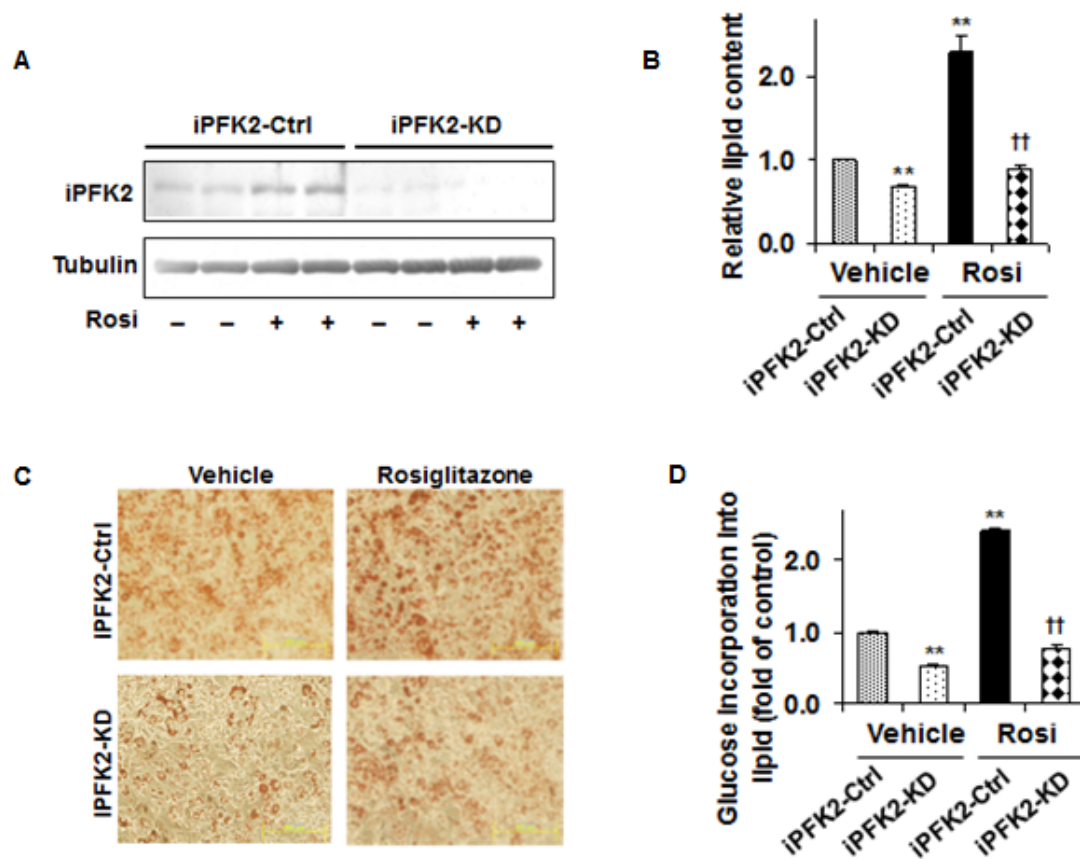


Figure 35. Knockdown of PFKFB3/iPFK2 lessens the effect of PPAR γ activation on stimulating adipocyte lipid accumulation. After differentiation for 6–8 days, stable iPFK2-KD and iPFK2-Ctrl adipocytes were treated with rosiglitazone (Rosi; 1 μ M) or vehicle (0.1% DMSO) for 48 h. Thereafter, the treated cells were subjected to the assays described under “Experimental Procedures.” A, adipocyte iPFK2 was determined using Western blot. B, representative images of adipocyte lipid content. Yellow bar, 500 μ m. For C and D, data are means \pm S.E. (error bars), $n = 4$. **, $p < 0.01$, iPFK2-KD/vehicle or iPFK2-Ctrl/rosiglitazone vs. iPFK2-Ctrl/vehicle; ††, $p < 0.01$, iPFK2-KD/Rosi vs. iPFK2-Ctrl/rosiglitazone. C, quantification of adipocyte lipid accumulation (arbitrary units). D, changes in the rate of glucose incorporation into lipid.

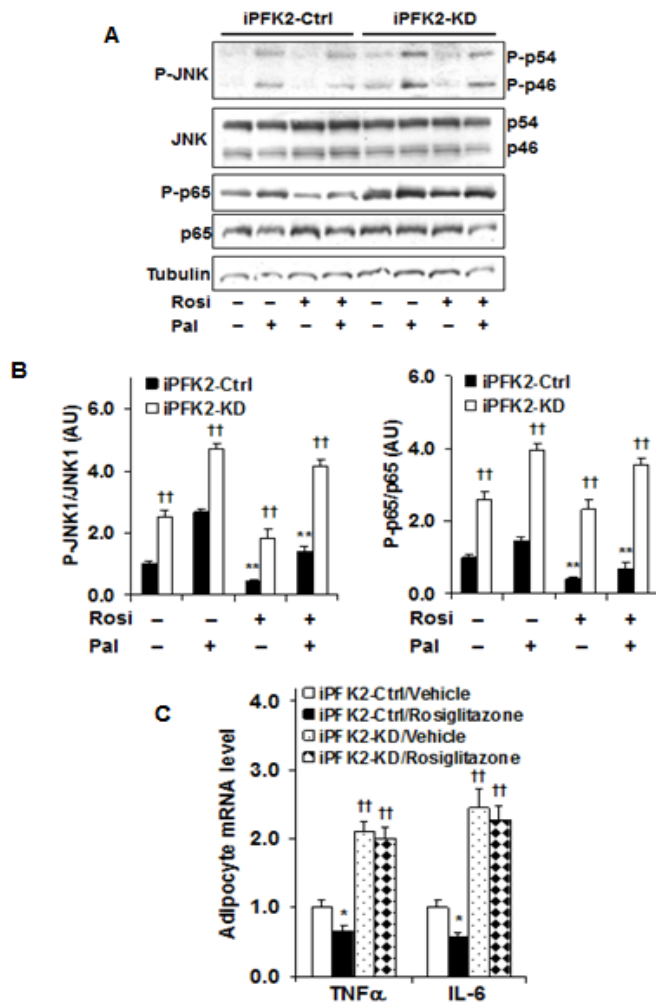


Figure 36. Knockdown of PFKFB3/iPFK2 diminishes the effects of PPAR γ activation on suppression of adipocyte inflammatory response. After differentiation for 6–8 days, stable iPFK2-KD and iPFK2-Ctrl adipocytes were treated with rosiglitazone (Rosi; 1 μ M) or vehicle (0.1% DMSO) for 48 h in the presence or absence of palmitate (Pal; 250 μ M) for the last 24 h. Thereafter, the treated cells were subjected to the assays described under “Experimental Procedures.” A, changes in inflammatory signaling were analyzed using Western blots. For B and C, data are means \pm S.E., n = 4. B, quantification of inflammatory signaling (arbitrary units). Left, phospho-JNK1 (P-JNK1)/JNK1; right, phospho-p65 (P-p65)/p65. **, p < 0.01, iPFK2-Ctrl treated with Rosi vs. iPFK2-Ctrl treated without Rosi in the presence or absence of Pal. ††, p < 0.01, iPFK2-KD vs. iPFK2-Ctrl under the same condition. For C, the expression of proinflammatory cytokines was measured using real-time RT-PCR. *, p < 0.05, iPFK2-Ctrl/rosiglitazone versus iPFK2-Ctrl/vehicle for the same gene. ††, p < 0.01, iPFK2-KD/vehicle vs. iPFK2-Ctrl/vehicle or iPFK2-KD/rosiglitazone vs. iPFK2-Ctrl/rosiglitazone for the same gene.

Knockdown of PFKFB3/iPFK2 Diminishes the Effects of PPAR γ Activation on Improvement of Adipocyte Function

Increased inflammatory response contributes to inappropriate expression of adipokines and decreased insulin signaling in adipocytes. To analyze the direct involvement of PFKFB3/iPFK2 in the effect of PPAR γ activation on adipocyte function, the mRNA levels of resistin and adiponectin as well as insulin signaling were determined in rosiglitazone- or vehicle-treated iPFK2-KD and iPFK2-Ctrl adipocytes. Compared with vehicle, treatment with rosiglitazone caused a decrease in the mRNA levels of resistin and an increase in the mRNA levels of adiponectin in iPFK2-Ctrl adipocytes (Fig. 37 A). Additionally, treatment with rosiglitazone brought about an increase in insulin-stimulated phosphorylation of Akt in iPFK2-Ctrl adipocytes (Fig. 37 B). However, these beneficial effects of rosiglitazone were diminished in iPFK2-KD adipocytes. Together, these data suggest that PFKFB3/iPFK2 is directly involved in the effect of PPAR γ activation on improving adipocyte function.

Inhibition of Fatty Acid Oxidation Restores the Effects of PPAR γ Activation on Both Suppression of Adipocyte Inflammatory Response and Stimulation of Adipocyte Insulin Signaling

PFKFB3/iPFK2 links fuel metabolism and inflammatory response in adipocytes via suppression of fatty acid oxidation-associated production of ROS. The extent to which PFKFB3/iPFK2 modulates the effect of PPAR γ activation on ROS production was determined. Compared with that in iPFK2-Ctrl adipocytes, the ROS production was

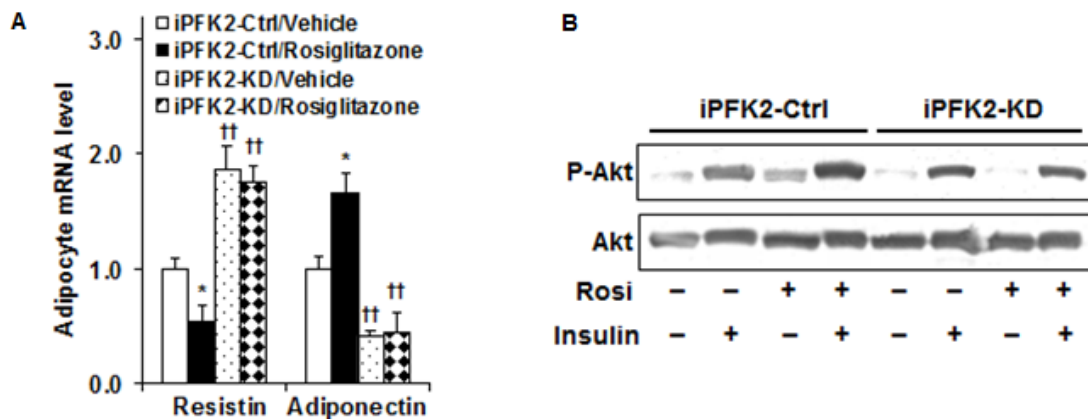


Figure 37. Knockdown of PFKFB3/iPFK2 diminishes the effects of PPAR γ activation on improving of adipocyte function. After differentiation for 6–8 days, stable iPFK2-KD and iPFK2-Ctrl adipocytes were treated with rosiglitazone (Rosi; 1 μ M) or vehicle (0.1% DMSO) for 48 h in the presence or absence of palmitate (Pal; 250 μ M) for the last 24 h. Thereafter, the treated cells were subjected to the assays described under “Experimental Procedures.” A, the expression of adipokines was measured using real-time RT-PCR. *, $p < 0.05$, iPFK2-Ctrl/rosiglitazone versus iPFK2-Ctrl/vehicle for the same gene. ††, $p < 0.01$, iPFK2-KD/vehicle vs. iPFK2-Ctrl/vehicle or iPFK2-KD/rosiglitazone vs. iPFK2-Ctrl/rosiglitazone for the same gene. B, adipocyte insulin signaling was analyzed using Western blot. Before harvest, the cells were incubated with or without insulin (100 nM) for 30 min. P-p46, phospho-p46.

higher in iPFK2-KD adipocytes under the basal condition (without palmitate) and was markedly increased in the palmitate-stimulated condition (Fig. 38 A). Upon treatment with rosiglitazone, the ROS production was low in iPFK2-Ctrl adipocytes and remained unchanged upon the addition of palmitate. However, in iPFK2-KD adipocytes, rosiglitazone treatment did not significantly decrease the basal ROS production and failed to blunt the palmitate-induced increase in ROS production (Fig. 38 A). It appears that PFKFB3/iPFK2-knockdown-associated increase in ROS production blunts the

beneficial effects of PPAR γ activation in iPFK2-KD adipocytes. Next, we determined the extent to which correction of excessive ROS production restores the effects of PPAR γ activation on inflammatory response and insulin signaling in iPFK2-KD adipocytes. Upon inhibition of fatty acid oxidation by etomoxir, palmitate-stimulated ROS production in iPFK2-KD adipocytes was decreased to a level comparable with that in untreated iPFK2-Ctrl adipocytes (Fig. 38 B). Under this condition, treatment with rosiglitazone brought about a decrease in the phosphorylation of JNK1 and NF- κ B p65 as well as the mRNA levels of TNF α and IL6 in iPFK2-KD adipocytes to their respective levels comparable with those in rosiglitazone-treated iPFK2-Ctrl adipocytes (Fig. 38, C and D). These effects did not occur in iPFK2-KD adipocytes in the absence of etomoxir (see above; Fig. 38, A–C). Additionally, upon supplementation of etomoxir, treatment with rosiglitazone increased the phosphorylation of Akt in iPFK2-KD adipocytes in the presence of palmitate (Fig. 38 E), which was also not observed in iPFK2-KD adipocytes incubated without etomoxir (see above; Fig. 38 E). Collectively, these data suggest that inhibition of excessive fatty acid oxidation restores the effects of PPAR γ activation on both suppression of adipocyte inflammatory response and stimulation of adipocyte insulin signaling in iPFK2-KD adipocytes.

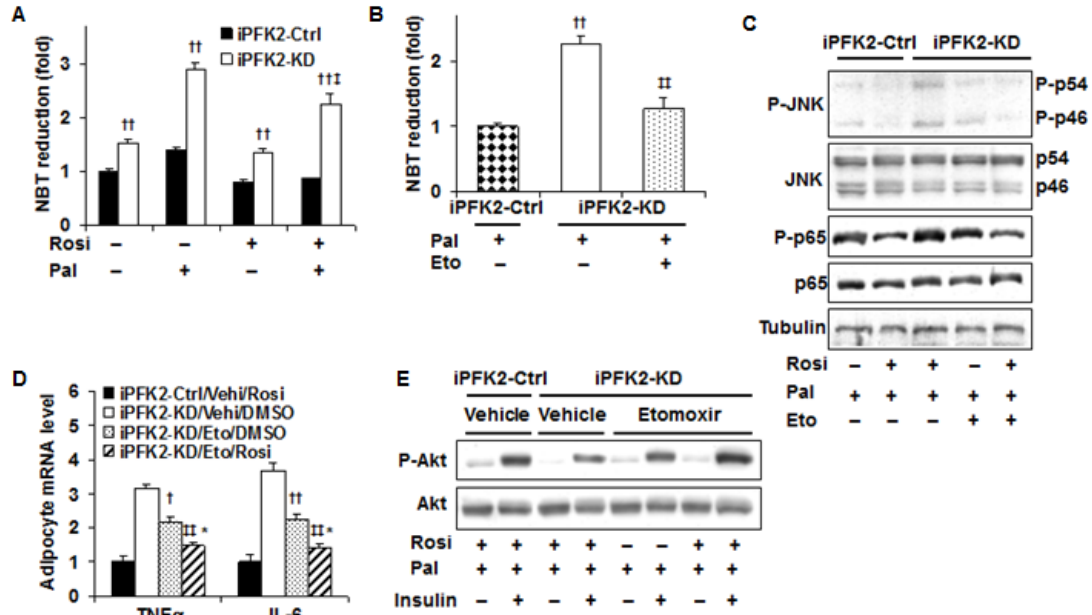


Figure 38. Inhibition of fatty acid oxidation restores the effects of PPAR γ activation on both suppression of adipocyte inflammatory response and stimulation of adipocyte insulin signaling. After differentiation for 6–8 days, stable iPFK2-KD and iPFK2-Ctrl adipocytes were treated with rosiglitazone (*Rosi*; 1 μ M) or vehicle (0.1% DMSO) for 48 h. In the last 24 h, the cells were incubated with or without etomoxir (*Eto*; 100 μ M) in the presence or absence of palmitate (*Pal*; 250 μ M) for 24 h. Thereafter, the treated cells were subjected to the assays described under “Experimental Procedures.” For A and B, the production of ROS was measured using the nitro blue tetrazolium assay. Data are means \pm S.E. (error bars), $n = 4$. A, $\dagger\dagger$, $p < 0.01$, iPFK2-KD vs. iPFK2-Ctrl under the same condition. \ddagger , $p < 0.05$, iPFK2-KD in the presence of palmitate vs. iPFK2-KD in the absence of palmitate under treatment with rosiglitazone. B, $\dagger\dagger$, $p < 0.01$ iPFK2-KD vs. iPFK2-Ctrl in the absence of etomoxir; $\ddagger\dagger$, $p < 0.01$ iPFK2-KD in the presence of etomoxir vs. iPFK2-KD in the absence of etomoxir. C, changes in adipocyte inflammatory signaling. D, changes in adipocyte expression of proinflammatory cytokines. Data are means \pm S.E. (error bars), $n = 4$. \dagger , $p < 0.05$; $\dagger\dagger$, $p < 0.01$, iPFK2-KD/etomoxir/DMSO vs. iPFK2-KD/vehicle (Vehi)/DMSO for the same gene. $\ddagger\dagger$, $p < 0.01$, iPFK2-KD/etomoxir/rosiglitazone vs. iPFK2-KD/Vehi/DMSO; *, $p < 0.05$, iPFK2-KD/etomoxir/rosiglitazone vs. iPFK2-KD/etomoxir/DMSO. E, changes in adipocyte insulin signaling. Before harvest, the cells were incubated with or without insulin (100 nM) for 30 min.

DISCUSSION

PFKFB3/iPFK2 has been identified as a target gene of PPAR γ since the finding that troglitazone, an early TZD, increases PFKFB3/iPFK2 expression in adipocytes [110, 156]. Two lines of evidence were obtained to further demonstrate the role of PPAR γ in controlling PFKFB3/iPFK2 expression. Notably, disruption of PPAR γ 2 decreased PFKFB3/iPFK2 expression, whereas PPAR γ 2 overexpression rescued this defect. In addition, agonist(s) for PPAR γ but not PPAR α and/or PPAR δ stimulated the expression of adipocyte PFKFB3/iPFK2, which was attributed to transcription activation of the promoter of PFKFB3. Interestingly, the metabolic phenotype of PFKFB3/iPFK2-disrupted mice was similar to that of PPAR γ 2-disrupted mice [120] and adipose tissue-specific PPAR γ -knock-out mice on an HFD [90]. This leads to the hypothesis that PFKFB3/iPFK2 is critically involved in the antidiabetic effect of PPAR γ activation. To test this hypothesis, using PFKFB3/iPFK2-disrupted mice and PFKFB3/iPFK2- and PEPCCK to rosiglitazone in PFKFB3^{+/-} mice was not sufficient to compensate for the PFKFB3/iPFK2 disruption-associated decrease in the ability of adipose tissue to store fat. A possible explanation directly linked to the biochemical properties of these enzymes is that PFKFB3/iPFK2 generates both acetyl-CoA and glycerol 3-phosphate as the required substrates for lipogenesis and triglyceride synthesis in adipocytes, whereas GyK and PEPCCK appear to only generate glycerol 3-phosphate via direct phosphorylation of glycerol [100] and through glyceroneogenesis [94], respectively. Also, it is possible that the stimulatory effects of GyK and PEPCCK on adipocyte/adipose

tissue fat storage were offset by the PFKFB3/iPFK2 disruption-associated increase in adipose tissue fatty acid oxidation; given that PFKFB3/iPFK2 has an indirect effect on suppression of adipocyte fatty acid oxidation. Based on the current data, the possibility that PFKFB3/iPFK2 disruption generated an environment that could not allow GlyK and/or PEPCK to stimulate fat storage cannot be ruled out and will be investigated by future study.

The contribution of PFKFB3/iPFK2 to the anti-diabetic effect of PPAR γ activation is also attributable to the suppressive effect of PFKFB3/iPFK2 on adipocyte inflammatory response. Treatment with rosiglitazone brought about a decrease in HFD-stimulated adipose mRNA levels of TNF α and IL-6 in wild-type mice but not in PFKFB3^{+/-} mice. Further, a direct role for PFKFB3/iPFK2 in mediating the anti-inflammatory effect of PPAR γ activation was confirmed in a cell culture system. In PFKFB3/iPFK2-knockdown 3T3-L1 adipocytes, rosiglitazone did not decrease palmitate-induced mRNA levels of TNF α and IL-6 as it did in control adipocytes. This PFKFB3/iPFK2 knockdown-associated defect resulted in an inefficiency of rosiglitazone to decrease adipose resistin mRNA levels and to increase adiponectin mRNA levels in both PFKFB3^{+/-} mice and PFKFB3/iPFK2-knockdown adipocytes, which indicated adipose tissue/adipocyte dysfunction [158-160] and contributed to the lack of anti-diabetic effect in rosiglitazone-treated PFKFB3^{+/-} mice.

In conclusion, these data support the involvement of PFKFB3/iPFK2 in the anti-diabetic effect of PPAR γ activation. This role of PFKFB3/iPFK2 is evidenced by the fact that the intact PFKFB3/iPFK2 allows rosiglitazone to increase fat storage in the

adipocytes/adipose tissue and to suppress adipocyte/adipose tissue inflammatory response. These results indicate that selective activation of adipocyte PFKFB3/iPFK2 may be a viable approach to generating the beneficial effects of PPAR γ activation in the treatment of type 2 diabetes.

CHAPTER V

THE ROLE OF PFKFB3/iPFK2 IN OVERNUTRITION-RELATED INTESTINE INFLAMMATION*

INTRODUCTION

Overnutrition contributes to systemic insulin resistance that increases the risk of metabolic disorders. There are many studies focusing on metabolic disorders in liver, adipose tissue and skeletal muscle. However, little studies investigate the role of the intestine in overnutrition-related metabolic disorders. The intestine is the organ for digestion, absorption and assimilation. Nutritional signals after food ingestion stimulate intestinal enterocytes to secrete hormones and cytokines. Liver, adipose tissue and skeletal muscle adjust their function to maintain systemic homeostasis when respond to those hormones and cytokines secreted by the intestine [64]. For example, incretin hormones facilitate glucose uptake by muscle and suppress the production of glucose in liver [62, 63]. The hormones secreted by the intestine can control not only the intestinal motility and satiety but also the glucose homeostasis [64]. In HFD-induced obesity and insulin resistance mouse models, many genes related to lipid metabolism are changed in

* Reprinted with permission from Guo, X., Li, H., Thomas, L.N., Sturino, J.M., and Wu, C. *Disruption of PFKFB3/iPFK2 blunts the suppressive effect of rosiglitazone on diet-induced intestine inflammatory response*. J Nutr Biochem, 2012. [Epub ahead of print] Copyright 2012 by Elsevier B.V.

the small intestine. Those fat-modulated genes are associated with insulin resistance [65]. In addition to digestion, absorption and assimilation, the intestine is also an organ for collection of microbes. There is a balance between the intestine and the intestinal microbiota. Failure to maintain the balance causes impairment of intestinal homeostasis, even systemic homeostasis [70]. Under overnutrition, gut microbiota are changed and promote the secretion of proinflammatory cytokines that are associated with the development and progression of insulin resistance and metabolic disorder [66, 71]. Mechanisms for the control of inflammation by microbiota could be due to that microbiota increase intestinal permeability [72, 73]. Therefore, the role of intestine in critically in regulating systemic homeostasis should be taken seriously.

Rosiglitazone is one of the thiazolidinediones (TZDs) that activate the nuclear receptor called Peroxisome Proliferator-activated receptor γ (PPAR γ). Activation of PPAR γ effectively improves systemic insulin sensitivity and lowers plasma glucose levels in both human patients and rodent models of type 2 diabetes [76-82]. PPAR γ is at high abundance in adipose tissue and associated with adipocyte differentiation [89, 161]. As such, adipose tissue is considered to be the primary organ for the anti-diabetic effect of PPAR γ activation. There are two mechanisms to explain the anti-diabetic effect of PPAR γ activation [45, 81]. First, PPAR γ activation by TZDs increases the capacity of fat storage in adipose tissue to reduce the circulating levels of free fatty acids [94-96, 100]. Second, PPAR γ activation suppresses the adipose tissue inflammatory response [58, 97] and regulates adipokine expression [98, 101]. In the past decade, the anti-inflammatory

effect of PPAR γ activation in intestine has been reported. Additionally, activation of PPAR γ is shown to be effective for decreasing the inflammation in certain intestinal diseases [162-164]. Considering this, there is a need to define a role for intestine in anti-diabetic effect of PPAR γ activation.

PFKFB3 is the gene that encodes for the inducible 6-phosphofructo-2-kinase (iPFK2) that is highly expressed in adipose tissue [110]. PFKFB3/iPFK2 generates fructose-2,6-bisphosphate (F26P₂), which is a powerful activator of 6-phosphofructo-1-kinase (6PFK1) to enhance glycolysis [111, 112]. This effect is involved in adipocyte lipogenesis and triglyceride synthesis [110]. It has previously been shown that PFKFB3/iPFK2 regulates diet-induced adiposity and systemic insulin resistance. For example, on an HFD, PFKFB3^{+/-} mice had a smaller increase in adiposity than wild type littermates. Compared with wild type littermates, however, PFKFB3^{+/-} mice exhibited increased severity of HFD-induced adipose tissue dysfunction, decreased insulin signaling, and increased inflammatory response. Therefore, PFKFB3/iPFK2 protects against diet-induced insulin resistance and adipose tissue inflammatory response, although contributing to adiposity. However, it is unknown whether the protection of PFKFB3/iPFK2 influences the inflammatory response in other organs or tissues such as the intestine. The present study provides evidence to support a role of adipose PFKFB3/iPFK2 in regulating diet-induced intestinal inflammatory response.

MATERIALS AND METHODS

Animal Experiments

All mice were maintained on a 12:12-h light-dark cycle (lights on at 06:00). For PFKFB3/iPFK2 distribution study, male wild-type C57BL/6J mice were fed ad libitum. At 12 – 14 weeks of age, mice were euthanized to collect tissue samples. In order to investigate the effect of diet on intestine, male wild-type C57BL/6J mice were fed with a high fat diet (HFD) (60% fat calories, 20% protein calories, and 20 carbohydrate calories) or a low fat diet (LFD) (10% fat calories, 20% protein calories, and 70 carbohydrate calories) for 12 weeks at the age of 5 – 6 weeks. Both diets are products of Research Diets, Inc (New Brunswick, NJ) and contain the same of amount of casein, L-cystein, cellulose, soybean oil, and minerals. However, the HFD contains much more lard and maltodextrin but much less sucrose and none corn starch compared to the LFD. At 12 – 14 weeks of age, mice were euthanized to collect tissue samples. In order to investigate the function of PFKFB3/iPFK2 in intestine, PFKFB3/iPFK2 disrupted mice were introduced. Homozygous disruption of PFKFB3/iPFK2 is embryonic lethal [133]. Thus, PFKFB3^{+/-} mice used in the present study. Considering that rosiglitazone lowers the levels of plasma glucose and improves insulin sensitivity only in diabetic mice, male PFKFB3^{+/-} and wild-type littermates (C57BL/6J background) were fed an HFD prior to treatment with rosiglitazone. It has been recently shown that feeding an HFD to PFKFB3^{+/-} mice exacerbates systemic insulin resistance and adipose tissue inflammatory response [165]. At the age of 5 - 6 weeks, mice were fed an HFD or an LFD for 12

weeks. During the last 4 weeks of the feeding regimen, HFD-fed mice were treated with rosiglitazone (10 mg/kg/day in PBS; Avandia tablets) or vehicle (PBS) via oral gavages. LFD-fed age-matched male mice were served as control and received no treatment. At the end of the feeding or treatment regimen, all mice were fasted for 4 h before sacrifice for collection of blood and tissue samples [135-137]. After anesthesia with ketamine/Xylene (100 mg/kg / 10 mg/kg body weight) via intraperitoneal injection, the abdomen was quickly opened. The intestine samples were frozen in liquid nitrogen and then stored at -80 °C for further study. All intestine samples were got rid of the digesta or feces. All study protocols were reviewed and approved by the Institutional Animal Care and Use Committee of Texas A&M University.

Determination of PFKFB3 mRNA and iPFK2 Amount

PFKFB3 mRNA and iPFK2 amount in the intestine were determined using real-time RT-PCR and Western blot, respectively, as described below.

RNA Isolation, Reverse Transcription, and Real-time PCR

The total RNA was isolated from frozen intestine (jejunum and ileum) samples. RNA isolation and real-time RT-PCR were conducted as previously described [137]. The mRNA levels were analyzed for TLR4, TNF α , and IL-6.

Western Blots

Lysates were prepared from intestine (jejunum and ileum) samples. Western blots were conducted as previously described [136, 137]. The levels of iPK2, JNK, phospho-JNK, NF- κ B p65 and phospho-p65 were analyzed.

Measurement of Microbiota Composition

Before and during treatment with rosiglitazone or PBS, fecal samples of HFD-fed mice were collected, pooled and homogenized. Total genomic DNA was isolated and subjected to real-time PCR using primers specific to bifidobacteria [166] and lactobacilli [167].

Evaluation of Systemic Insulin Sensitivity

Plasma levels of glucose and insulin were measured as previously described and used to calculate homeostasis model assessment of insulin resistance (HOMA-IR), an indicator of systemic insulin resistance, using the following equation: $\text{HOMA-IR} = \text{basal glucose (mmol/L)} \times \text{basal insulin (mU/L)} / 22.5$.

Statistical Methods

Numeric data are presented as means \pm SE (standard error). Statistical significance was assessed by unpaired, two-tailed ANOVA or Student's *t* test. Differences were considered significant at the two-tailed $P < 0.05$.

RESULTS

PFKFB3/iPFK2 is Expressed Abundantly in the Intestine

The expression profile of PFKFB3/iPFK2 was determined in various tissues in wild-type mice. Among the key tissues that are involved in the regulation of systemic insulin sensitivity and metabolic homeostasis, PFKFB3/iPFK2 is expressed at high levels in the intestine and white adipose tissue but expressed at very low levels in the liver, muscle, and brown adipose tissue (Fig. 39). PFKFB3/iPFK2-associated metabolic changes are due primarily to alteration of PFKFB3/iPFK2 in the white adipose tissue.

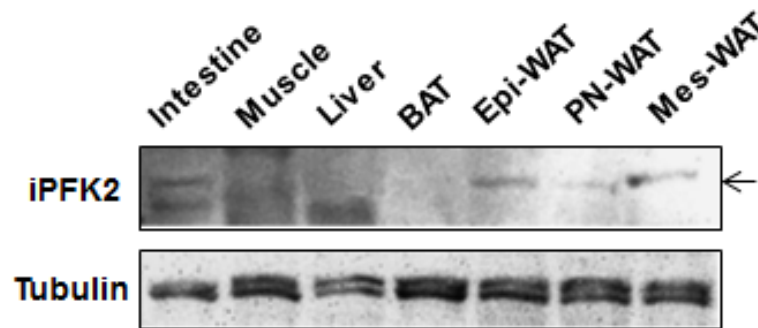


Figure 39. PFKFB3/iPFK2 expression in key metabolic tissues. Male wild-type C57BL/6J mice were fed ad libitum. At 12–14 weeks of age, mice were euthanized for collection of tissue samples. Tissue lysates were prepared to determine the amount of iPFK2 using Western blot analyses. BAT, brown adipose tissue; Epi-WAT, epididymal white adipose tissue; PN, perinephric; Mes, mesenteric.

The role of PFKFB3/iPFK2 in white adipose tissue was described in previous study. These data suggest that except to the white adipose tissue, the intestine also plays a role in PFKFB3/iPFK2-associated metabolic changes.

Diet Effects the Expression of PFKFB3/iPFK2 in the Intestine

To determine the role of diet in the expression of intestinal PFKFB3/iPFK2, the protein levels of intestinal PFKFB3/iPFK2 in LFD-fed and HFD-fed wild-type mice were measured. Compared to the LFD-fed wild-type mice, the levels of intestinal PFKFB3/iPFK2 were significantly higher in HFD-fed wild-type mice (Fig. 40). This data suggests that HFD feeding increases the expression of PFKFB3/iPFK2 in the intestine. This increase in intestine PFKFB3/iPFK2 expression appears to be a defensive response, given a critical role for PFKFB3/iPFK2 in protecting against diet-induced intestine inflammatory response.

HFD Feeding Induces Intestine Inflammatory Response

Intestine inflammatory response was examined. Compared with controls, the phosphorylation of JNK1 in intestine of HFD-fed mice was increased (Fig. 41 A), although the phosphorylation of NF- κ B was undetectable in mice on either an HFD or LFD (data not shown). Additionally, in HFD-fed mice, intestine mRNA levels of TNF α and IL-6 were significantly higher than their respective levels in controls (Fig. 41 B). These results demonstrate an increase in intestine inflammatory response in HFD-fed mice.

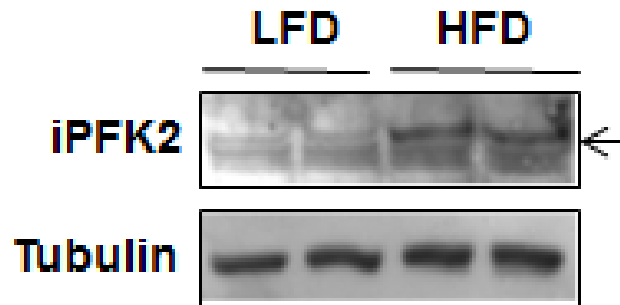


Figure 40. Diet effects the expression of PFKFB3/iPFK2 in the intestine. Male wild-type C57BL/6J mice, at 5–6 weeks of age, were fed an LFD or HFD for 12 weeks. Intestine lysates were subjected to Western blot analyses to measure intestine amount of iPFK2.

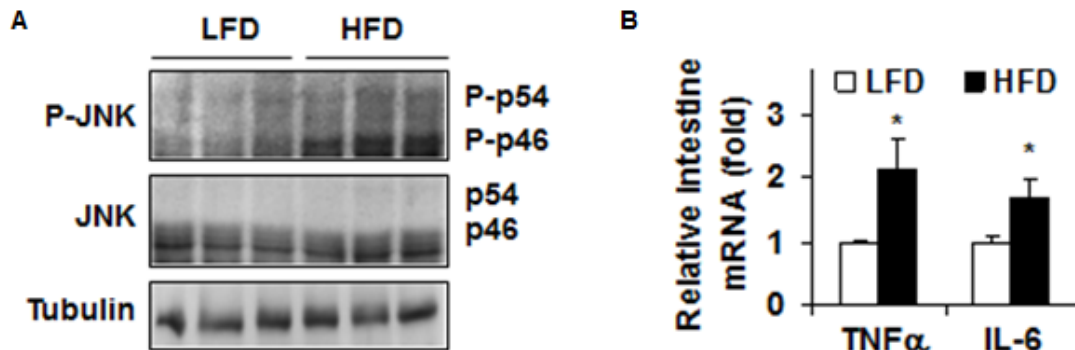


Figure 41. Effects of HFD feeding on intestine inflammatory responses. Male wild-type C57BL/6J mice, at 5–6 weeks of age, were fed an LFD or HFD for 12 weeks. (A) Intestine lysates were subjected to Western blot analyses to measure intestine amount of JNK and phospho-JNK. (B) The total RNA of intestine was prepared to determine the expression of proinflammatory cytokines using real-time RT-PCR. Data are means \pm S.E., n=4. *P < 0.05 HFD vs. LFD for the same gene.

PFKFB3/iPFK2 Disruption Blunts Dietary Response of Intestine iPFK2

The response of intestine PFKFB3/iPFK2 to HFD feeding was examined in PFKFB3^{+/-} mice. Heterozygous PFKFB3 disruption was confirmed using PCR analyses of genomic DNA (Fig. 42 A). On an LFD, intestine iPFK2 amount in PFKFB3^{+/-} mice was lower than in wild type littermates (C57BL/6J background) (Fig. 42 B), further demonstrating PFKFB3/iPFK2 disruption. Upon feeding an HFD, PFKFB3^{+/-} mice did not exhibit an increase in intestine iPFK2 amount as did wild-type mice. Thus, intact PFKFB3/iPFK2 appears to be required for a defensive increase in intestine PFKFB3/iPFK2 in response to HFD feeding.

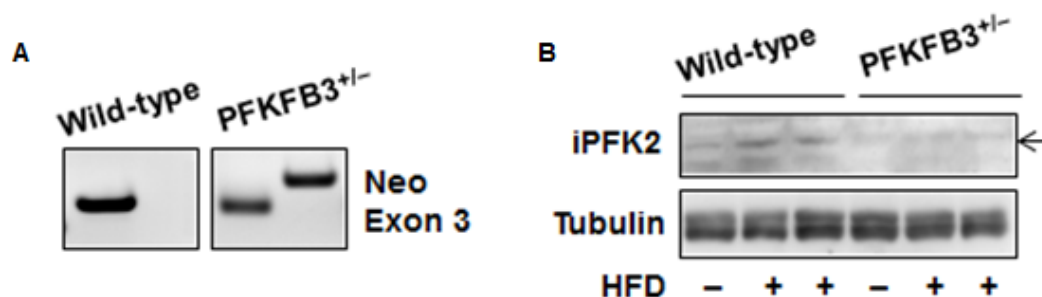


Figure 42. Effects of PFKFB3/iPFK2 disruption on dietary response of intestine iPFK2. (A) Validation of heterozygous PFKFB3/iPFK2 disruption. Genomic DNA of PFKFB3^{+/-} mice and wild-type littermates were subjected to PCR analyses using an exon 2-specific primer with an exon 3-specific primer (WT) or a neomycin-specific primer (Neo). (B) Male PFKFB3^{+/-} mice and wild-type littermates, at 5–6 weeks of age, were fed an LFD or HFD for 12 weeks. Intestine lysates were prepared to determine the amount of iPFK2 using Western blot analyses.

PFKFB3/iPFK2 Disruption Exacerbates HFD-induced Intestine Inflammatory Response and Partially Blunts the Effects of PPAR γ Activation

The effect of PFKFB3/iPFK2 disruption on diet-induced intestine inflammatory response was examined. Compared with HFD-fed wild type littermates, HFD-fed PFKFB3^{+/-} mice exhibited an increase in the mRNA levels of intestine TLR4 (Fig. 43 A), a receptor whose activation leads to increased proinflammatory responses. In addition, the phosphorylation of JNK1 and NF- κ B p65, two key signaling pathways that mediate proinflammatory responses, in intestine of HFD-fed PFKFB3^{+/-} mice was much greater than that in controls (Fig. 43 B). Consistent with increased signaling through proinflammatory pathways, the mRNA levels of intestine TNF α and IL-6 in HFD-fed PFKFB3^{+/-} mice were higher than their respective levels in controls (Fig. 43 C). These results, in combination, suggest a protective role for PFKFB3/iPFK2 in diet-induced intestine inflammatory response.

PPAR γ has a protective role in intestine inflammation[168]. The effect of PPAR γ activation on diet-induced intestine inflammatory response was examined. Upon treatment with rosiglitazone, the mRNA levels of TLR4 (Fig. 43 A), the phosphorylation of JNK1 (Fig. 43 B), and the mRNA levels of TNF α and IL-6 (Fig. 43 C) were decreased in intestine of HFD-fed wild-type littermates and, to a much lesser degree, in intestine of HFD-fed PFKFB3^{+/-} mice. Additionally, intestine NF- κ B p65 phosphorylation remained high in HFD-fed PFKFB3^{+/-} mice compared with that in HFD-fed wild-type littermates after treatment with rosiglitazone (Fig. 43 B). Together,

these results suggest that PFKFB3/iPFK2 disruption partially blunts the effect of PPAR γ activation on suppressing HFD-induced intestine inflammatory response.

PFKFB3/iPFK2 Disruption Decreases Intestine Proliferation of Lactobacilli in HFD-fed Mice in Response to PBS and/or Rosiglitazone Treatment

Intestinal microbiotas not only control the inflammatory response in intestine but also critically regulate systemic insulin sensitivity [67-69]. The fecal samples of HFD-fed mice were analyzed in order to determine changes in proliferation of beneficial intestinal bacteria, specifically those belonging to the genera *Lactobacillus* and *Bifidobacterium*. Compared with controls, the proliferation of *Lactobacillus* in HFD-fed PFKFB3+/- mice was decreased upon treatment with either rosiglitazone or PBS (Fig. 44 A). However, bifidobacteria did not show significant differences among all four groups of mice (Fig. 44 B). These results suggest that oral dosing does not affect the proliferation of lactobacilli in wild-type animals, however oral dosing decreases their proliferation when PFKFB3/iPFK2 is disrupted. Furthermore, treatment with rosiglitazone has a limited role in altering intestine proliferation of *Lactobacillus* and *Bifidobacterium* in HFD-fed mice.

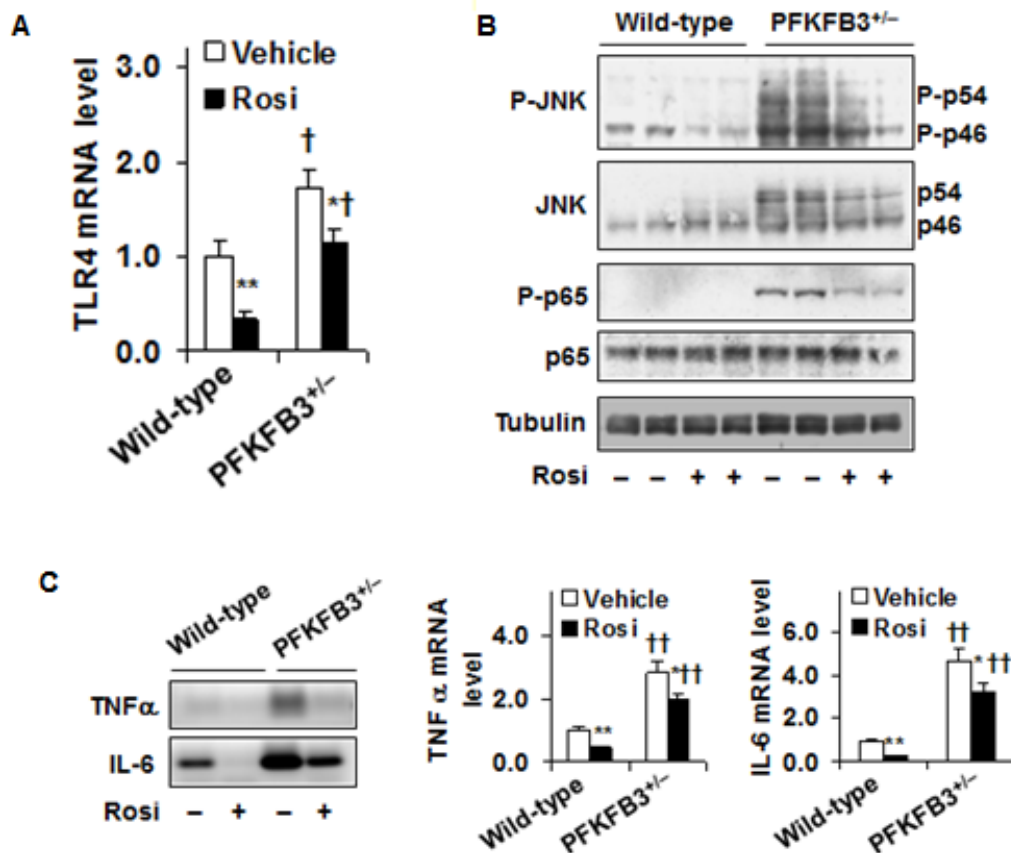


Figure 43. Involvement of PFKFB3/iPFK2 in the effect of PPAR γ activation on diet-induced intestine inflammatory response. Male PFKFB3^{+/-} mice and wild-type littermates, at 5–6 weeks of age, were fed an HFD for 12 weeks and treated with rosiglitazone (Rosi, 10 mg/kg/day in PBS) or vehicle (PBS) orally for the last 4 weeks of the feeding regimen. (A) Intestine mRNA levels of TLR4 were quantified using real-time RT-PCR. (B) Intestine inflammatory signaling. Intestine lysates were prepared to determine the amount and phosphorylation states of JNK and NF- κ B p65 using Western blot analyses. (C) Intestine mRNA levels of TNF α and IL-6 were quantified using real-time RT-PCR. Left panel, representative PCR products; and right two panels, relative intestine mRNA levels. For panels (A) and (C), numeric data are means \pm S.E.; n=4–6. *P < 0.05 and **P < 0.01, rosiglitazone vs. vehicle for the same genotype; † P < 0.05 and ††P < 0.01, PFKFB3^{+/-} vs. wild type for the same treatment (rosiglitazone or vehicle).

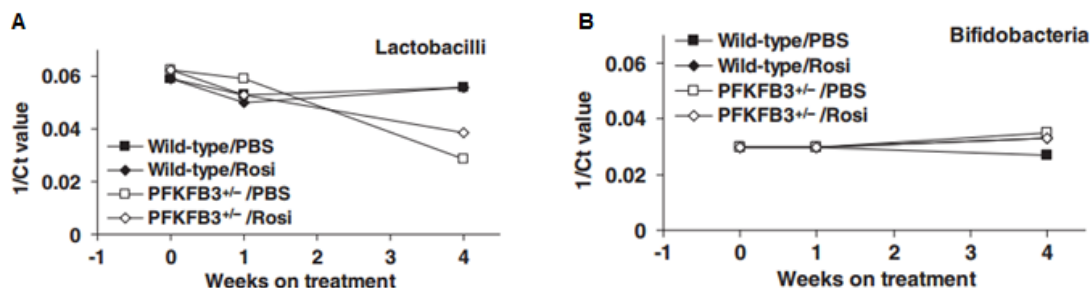


Figure 44. Alterations of intestine microbiota composition. Male PFKFB3^{+/-} mice and wildtype littermates, at 5–6 weeks of age, were fed an HFD for 12 weeks and treated with rosiglitazone (Rosi, 10 mg/kg/day in PBS) or vehicle (PBS) orally for the last 4 weeks of the feeding regimen. Before, during and after treatment with rosiglitazone or PBS, fecal samples of HFD-fed mice were collected, pooled and homogenized. Total genomic DNA was isolated and subjected to quantitative real-time PCR using primers specific to *Lactobacillus* (A) and *Bifidobacterium* (B). Ct, cycle threshold.

PFKFB3/iPFK2 Disruption Blunts the Insulin-sensitizing Effect of PPAR γ Activation

Feeding an HFD to mice induces intestine inflammatory response, which contributes to the development of systemic insulin resistance [169]. Upon treatment with rosiglitazone, HFD-fed wild-type mice displayed a marked decrease in HOMA-IR (Fig. 45), an indicator of insulin resistance. Significantly, the decrease in HOMA-IR in HFD-fed and rosiglitazone-treated wild-type mice was accompanied by decreased intestine inflammatory response as described above (Fig. 43). In contrast, treatment with rosiglitazone only caused an insignificant decrease in HOMA-IR in HFD-fed PFKFB3^{+/-} mice (Fig. 45), which was accompanied by increased intestine inflammatory response.

The latter was at a much greater degree than that in HFD-fed and rosiglitazone-treated wild-type mice. These results, in combination, indicate a positive correlation between systemic insulin resistance and intestine inflammatory response, which is regulated by PFKFB3/iPFK2.

DISCUSSION

In wild-type mice, feeding an HFD alters the fat-regulatory genes in the intestine, which contributes to systemic insulin resistance [65]. In PFKFB3^{+/-} mice,

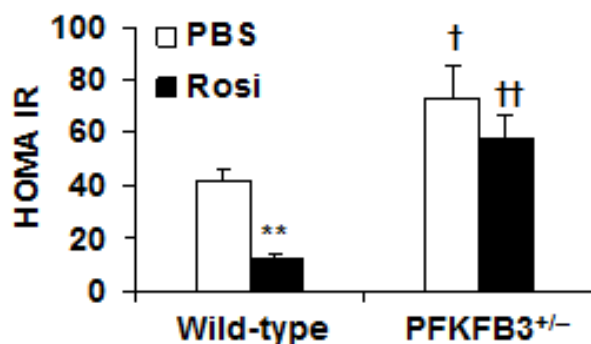


Figure 45. PFKFB3/iPFK2 regulation of systemic insulin sensitivity. Male PFKFB3^{+/-} mice and wild-type littermates, at 5–6 weeks of age, were fed an HFD for 12 weeks and treated with rosiglitazone (Rosi, 10 mg/kg/day in PBS) or vehicle (PBS) orally for the last 4 weeks of the feeding regimen. At the end of feeding/treatment regimen, blood samples were collected and used to measure plasma levels of glucose and insulin. HOMA-IR was calculated using the following equation: HOMA-IR=basal glucose (mmol/L)×basal insulin (mU/L)/22.5. Data are means ± S.E.; n=4–6. **P < 0.01, rosiglitazone vs. vehicle for the same genotype; † P < 0.05 and ††P < 0.01, PFKFB3^{+/-} vs. wild type for the same treatment (rosiglitazone or vehicle).

feeding an HFD caused a much greater increase in the severity of HFD-induced intestine inflammatory response and systemic insulin resistance. PFKFB3/iPFK2 has been identified as a target gene of PPAR γ because activation of PPAR γ increases PFKFB3/iPFK2 expression in adipocyte [110, 156]. In addition to adipose tissue, the intestine is another organ that abundantly expresses PFKFB3/iPFK2. Therefore, activation of PPAR γ may play a role in intestinal PFKFB3/iPFK2 expression. Also, PFKFB3/iPFK2 may be involved in the anti-diabetic effect of PPAR γ activation. In the present study, PFKFB3/iPFK2-disrupted mice were used to determine the extent to which PFKFB3/iPFK2 accounts for the anti-diabetic effect of PPAR γ activation. Treatment with rosiglitazone brought about a decrease in HFD-induced intestinal mRNA levels of TLR 4, TNF α and IL-6, and the phosphorylation of JNK1 in wild-type mice, but a partial decrease in inflammatory markers in PFKFB3^{+/-} mice. Additionally, the phosphorylation of intestinal JNK1 remained high in PFKFB3^{+/-} mice compared with that in wild-type littermates after treatment with rosiglitazone. Together, these data indicate that PFKFB3/iPFK2 plays an important role in regulating HFD-induced intestinal inflammatory response and systemic insulin resistance.

In summary, activation of intestinal PFKFB3/iPFK2 may be an approach to reversing overnutrition related intestinal inflammation and systemic insulin resistance, thereby generating the beneficial effects for the treatment of type 2 diabetes.

CHAPTER VI

SUMMARY AND CONCLUSIONS

SUMMARY

In wild-type mice, adiposity induced by overnutrition is a key contributor of systemic insulin resistance. However, in PFKFB3^{+/-} mice, although bringing about a much smaller increase in adiposity, feeding an HFD caused a much greater increase in the severity of HFD-induced adipose tissue dysfunction and systemic insulin resistance than in wild-type littermates. These changes were attributed to the increased adipose tissue inflammatory response, which was evidenced by higher levels of proinflammatory cytokines in both isolated adipose tissue macrophages and adipocytes in PFKFB3^{+/-} mice. Consistently, in cultured adipocytes, knockdown of PFKFB3/iPFK2 caused a decrease in lipid accumulation and an increase in the status of oxidative stress, which were accompanied by enhanced inflammatory signaling, increased mRNA levels of TNF α and IL-6, and decreased insulin signaling. PFKFB3/iPFK2 plays a novel and unique role in regulating HFD-induced adipose tissue dysfunction and systemic insulin resistance in a manner independent of adiposity.

The unique role of PFKFB3/iPFK2 in dissociating HFD-induced adiposity and adipose tissue dysfunction is attributed, at a large extent, to the metabolic properties of PFKFB3/iPFK2. Notably, PFKFB3/iPFK2 stimulates adipocyte glycolysis [110]. An

increase in PFKFB3/iPFK2-stimulated glycolysis not only provides lactate and pyruvate (which are converted into acetyl-CoA and used for lipogenesis to provide free fatty acids), but also increases the production of dihydroxyacetone phosphate, which is converted into glycerol-3-phosphate as a required substrate for adipocyte triglyceride synthesis. Upon disruption of PFKFB3/iPFK2, both glycolysis and glycolysis-derived lipogenesis and triglyceride synthesis are impaired, which is supported by the data that PFKFB3/iPFK2-knockdown adipocytes exhibited a decrease in the incorporation of glucose into lipid, and thereby adipocyte lipid accumulation. This contributes to a smaller gain in adiposity in PFKFB3^{+/-} mice after HFD feeding. Importantly, the impairment in using glucose as a fuel due to disruption of PFKFB3/iPFK2 likely causes a compensatory increase in fatty acid oxidation, which is supported by increased expression of CPT1, as well as PPAR γ and PGC1 in adipose tissue of PFKFB3^{+/-} mice. The compensatory increase in fatty acid oxidation not only contributes to a smaller gain in adiposity in PFKFB3^{+/-} mice after HFD feeding, but more importantly, appears to trigger oxidative stress. In support of this concept, PFKFB3/iPFK2-knockdown adipocytes exhibited an increase in the production of ROS under both basal and palmitate-stimulated conditions. Furthermore, inhibition of fatty acid oxidation by etomoxir caused a marked decrease in palmitate-stimulated production of ROS in PFKFB3/iPFK2-knockdown adipocytes. In agreement with the role oxidative stress in disturbing adipokine expression and initiating the inflammatory response in adipocytes [45], the increased production of ROS in PFKFB3/iPFK2-knockdown adipocytes was associated, on the one hand, with inappropriately altered expression of resistin and

adiponectin, and on the other hand, with increased phosphorylation states of JNK and NF κ B p65 and increased mRNA levels of TNF α and IL-6 in PFKFB3/iPFK2-knockdown adipocytes. Apparently, PFKFB3/iPFK2 links nutrient metabolism and adipocyte function.

The role of adipose tissue dysfunction in causing systemic insulin resistance has been well documented [125, 126]. Indeed, this role is illustrated by at least two plausible mechanisms [45-48]. In the first mechanism, adipose tissue dysfunction causes an increase in the production of free fatty acids, resistin, and retinol binding protein 4 (RBP4) and a decrease in the production of adiponectin. These factors are carried to the insulin-sensitive tissues including the liver and skeletal muscle through circulation to impair insulin signaling and ultimately bring about systemic insulin resistance [130-132, 170]. In the second mechanism, adipose tissue-derived proinflammatory cytokines are similarly carried to insulin-sensitive tissues through circulation to directly impair insulin signaling in the tissues [128, 171, 172]. In the present study, PFKFB3^{+/-} mice exhibited an increase in the severity of HFD-induced systemic insulin resistance and glucose intolerance. This was attributed, at least in part, to increased adipose tissue dysfunction, as evidenced by increased adipose tissue lipolysis, inappropriate adipokine expression, and decreased insulin signaling, as well as increased expression of proinflammatory cytokines including TNF α and IL-6 in the adipose tissue. These data support a pivotal role for PFKFB3/iPFK2 in protecting against HFD-induced adipose tissue dysfunction, and thereby systemic insulin resistance. Moreover, because PFKFB3/iPFK2 disruption-associated changes in adipose tissue dysfunction were nearly identical to those in

PFKFB3/iPFK2-knockdown adipocytes, PFKFB3/iPFK2 in adipocytes is thereby responsible largely for the regulation of adipose tissue function. It should be pointed out that PFKFB3/iPFK2 is also expressed in tissues other than adipose tissue [133]. Thus, a possible role for PFKFB3/iPFK2 in non-adipose tissue in regulating systemic insulin sensitivity cannot be ruled out.

It is also a novel finding that disruption of PFKFB3/iPFK2 exacerbated HFD-induced adipose tissue inflammatory response, which was also independent of adiposity. Additionally, the increased adipose tissue inflammatory response was independent of macrophage accumulation in adipose tissue. In a generally accepted concept, HFD-induced adipose tissue inflammatory is characterized by increased production of proinflammatory cytokines, which is positively correlated with macrophage infiltration [125, 126]. However, several lines of new evidence suggest that the inflammatory status of macrophages is more important than the number of macrophages in terms of controlling the production of proinflammatory cytokines in the adipose tissue. For example, mice that lack PPAR γ in macrophages exhibit fewer macrophages in adipose tissue but have higher mRNA levels of IL-6 than wild-type control mice [173]. In contrast, treatment with rosiglitazone, a PPAR γ agonist, increases the abundance of macrophages in adipose tissue, but decreases the production of proinflammatory cytokines such as IL-18 [97]. Considering this, disruption of PFKFB3/iPFK2 appears to increase the inflammatory activity of the infiltrated macrophages. This notion is indeed supported by the data that the mRNA levels of TNF α and IL-6 were higher in macrophages isolated from PFKFB3^{+/-} mice than in wild-type littermates. Therefore,

disruption of PFKFB3/iPFK2 exacerbates HFD-induced adipose tissue inflammatory response in a manner depending on increasing macrophage inflammatory status rather than macrophage infiltration. However, further study is required to elucidate the underlying mechanisms by which disruption of PFKFB3/iPFK2 blunts HFD-induced macrophage infiltration into adipose tissue. In gain-of-function study, adipocyte-specific PFKFB3 over-expression increased adiposity but suppressed overnutrition induced adipose tissue inflammatory response and improved insulin sensitivity.

PFKFB3/iPFK2 has been identified as a target gene of PPAR γ since the finding that troglitazone, an early TZD, increases PFKFB3/iPFK2 expression in adipocytes [110, 156]. At the integrative level, the metabolic phenotype of PFKFB3/iPFK2-disrupted mice was similar to that of PPAR γ 2-disrupted mice [120] and adipose tissue-specific PPAR γ -knock-out mice on an HFD [90]. This leads to the hypothesis that PFKFB3/iPFK2 is critically involved in the antidiabetic effect of PPAR γ activation. Upon treatment of PFKFB3^{+/-} mice with rosiglitazone, PFKFB3/iPFK2 appears to be needed for PPAR γ activation to fully suppress diet-induced intestine inflammatory response. Thus, iPFK2 is needed for the anti-diabetic effect PPAR γ activation.

The contribution of PFKFB3/iPFK2 to the anti-diabetic effect of PPAR γ activation is also attributable to the suppressive effect of PFKFB3/iPFK2 on adipocyte inflammatory response. Treatment with rosiglitazone brought about a decrease in HFD-stimulated adipose mRNA levels of TNF α and IL-6 in wild-type mice but not in PFKFB3^{+/-} mice. Further, a direct role for PFKFB3/iPFK2 in mediating the anti-inflammatory effect of PPAR γ activation was confirmed in a cell culture system. In

PFKFB3/iPFK2-knockdown 3T3-L1 adipocytes, rosiglitazone did not decrease palmitate-induced mRNA levels of TNF α and IL-6 as it did in control adipocytes. This PFKFB3/iPFK2 knockdown-associated defect resulted in an inefficiency of rosiglitazone to decrease adipose resistin mRNA levels and to increase adiponectin mRNA levels in both PFKFB3^{+/-} mice and PFKFB3/iPFK2-knockdown adipocytes, which indicated adipose tissue/adipocyte dysfunction [158-160] and contributed to the lack of anti-diabetic effect in rosiglitazone-treated PFKFB3^{+/-} mice.

In the adipose tissue, both macrophages and adipocytes are key determinants of overnutrition-induced adipose tissue inflammatory response[126, 174]. Attention has been increasingly paid to the effect of PPAR γ activation on suppression of the proinflammatory function of macrophages [175, 176]. Treatment with rosiglitazone effectively reduced the phosphorylation of JNK1 and NF- κ B p65 in control adipocytes but not in PFKFB3/iPFK2-knockdown adipocytes. This is consistent with the observation that rosiglitazone blunted palmitate-induced ROS production in control adipocytes but not in PFKFB3/iPFK2-knockdown adipocytes. When PFKFB3/iPFK2 disruption-associated excessive fatty acid oxidation was brought down with etomoxir treatment, rosiglitazone was able to decrease inflammatory response and stimulate insulin signaling in PFKFB3/iPFK2-knockdown adipocytes. Clearly, PFKFB3/iPFK2 contributes to the anti-inflammatory effect of PPAR γ activation through a mechanism involving suppression of excessive fatty acid oxidation in adipocytes.

PFKFB3/iPFK2 disruption exacerbates diet-induced adipose tissue inflammatory response. At the cellular level, PFKFB3/iPFK2 disruption-associated increase in fatty

acid oxidation leads to increased production of ROS and oxidative stress, thereby triggering adipocyte inflammatory response. This mechanism may also exist in intestine, given that PFKFB3/iPFK2 is abundantly expressed in intestine. In the present study, intestine iPFK2 amount in PFKFB3/iPFK2-disrupted mice was lower than that in wild-type mice and did not respond to HFD feeding as did intestine iPFK2 in wild-type mice in a defensive way. Furthermore, intestine iPFK2 amount negatively correlated with the degree of intestine inflammatory response. Because of this, it appears to be clear that intact PFKFB3/iPFK2 is required for full protection of overnutrition-induced intestine inflammatory responses. At this point, however, the extent to which the PFKFB3/iPFK2 in intestine cells, in particular the PFKFB3/iPFK2 in intestinal epithelial cells, protects against diet-induced intestine inflammatory response is unknown. What should also be noted is that adipose tissue inflammatory response is elevated in PFKFB3/iPFK2-disrupted mice. Of importance, the status of adipose tissue inflammatory response determines the outcome of inflammatory responses in distal tissues including the liver. As such, a possible contribution of elevated adipose tissue inflammation to an increase in diet-induced intestine inflammatory response in PFKFB3/iPFK2-disrupted mice cannot be ruled out. Considering this, there may exist a vicious cycle for inflammatory responses between adipose tissue and intestine, regardless of how an initiator triggers inflammatory responses.

The essential role for PFKFB3/iPFK2 in protecting against diet induced intestine inflammatory response is further supported by the involvement of PFKFB3/iPFK2 in the anti-inflammatory effect of PPAR γ activation. As a target gene of PPAR γ ,

PFKFB3/iPFK2 is stimulated by TZDs [110, 156]. Of significance, intact PFKFB3/iPFK2 is needed for actions of active PPAR γ on channeling fatty acids to triglyceride synthesis to reduce excessive fatty acid oxidation associated production of ROS, thereby suppressing inflammatory signaling through the JNK1 and NF- κ B pathways and decreasing proinflammatory cytokine expression in adipocytes/adipose tissue. In addition to causing an increase in major intestine proinflammatory indicators, PFKFB3/iPFK2 disruption also partially blunted the effect of rosiglitazone on suppressing diet-induced intestine inflammatory response. These changes in intestine inflammatory response were nearly identical to those observed in adipose tissue in PFKFB3/iPFK2-disrupted mice upon treatment with rosiglitazone. Because of this, it is conceivable that intact PFKFB3/iPFK2 is needed for PPAR γ activation to suppress overnutrition-induced intestine inflammatory response. On the other hand, although PPAR γ activation brought about an anti-inflammatory effect in intestine in a manner consistent with that reported previously[168], suppressing diet-induced adipose tissue inflammatory response may be a prerequisite for PPAR γ activation to suppress diet-induced intestine inflammatory response, given the primary role played by adipose tissue in the actions of PPAR γ activation. Indeed, failure of rosiglitazone in fully suppressing diet-induced intestine inflammatory response reported herein was accompanied by defects in actions of rosiglitazone on reserving adipose tissue inflammatory response in PFKFB3/iPFK2-disrupted mice.

Suppressing inflammatory responses by active PPAR γ underlies the insulin-sensitizing and antidiabetic effects of TZDs. The degree of intestine inflammatory

response in rosiglitazone- and/or control-treated PFKFB3/iPFK2-disrupted mice and wild-type mice positively correlated with insulin resistance, indicated by HOMA-IR results. This observation argues in favor of the notion that PFKFB3/iPFK2 protection of diet-induced intestine inflammatory response is of importance to systemic insulin sensitivity and glucose homeostasis, as well as insulin sensitization brought about by PPAR γ activation. As discussed before, there may exist a vicious cycle for inflammatory responses between adipose tissue and intestine during overnutrition. Considering this, intact PFKFB3/iPFK2 likely enables PPAR γ activation to suppress inflammatory responses in both adipose tissue and intestine to achieve a systemic insulin sensitizing effect. In other words, intact PFKFB3/iPFK2 enables PPAR γ activation to suppress intestine inflammatory response to contribute to systemic insulin sensitization by working with or without PPAR γ suppression of adipose tissue inflammatory response in a manner involving PFKFB3/iPFK2.

In intestine, microbiota not only controls energy absorption but also critically regulates inflammatory responses of intestine cells [64, 66, 73]. New evidence further demonstrates that interactions between HFD and microbiota promote inflammation in small intestine, which precedes and correlates with obesity and insulin resistance. In the present study, HFD-fed PFKFB3/iPFK2-disrupted mice showed a decrease in the proliferation of intestinal lactobacilli in response to treatment of either rosiglitazone or PBS compared with HFD-fed wild-type mice. These results, on the one hand, suggest that PFKFB3/iPFK2 disruption creates an intestinal environment that allows oral dosing to alter the composition of sensitive microorganism. On the other hand, treatment with

rosiglitazone has a limited role in altering the proliferation of intestinal *Lactobacillus*. Although it is unknown whether or not decreased proliferation of *Lactobacillus* contributes to an increase in intestine inflammatory response in PFKFB3/iPFK2-disrupted mice, a potential role for PFKFB3/iPFK2 in modulating the interactions between intestinal microorganism and intestine cells could serve as additional mechanism(s) by which PFKFB3/iPFK2 regulates intestine inflammatory response. This point is worthy of further investigation.

CONCLUSIONS

The present study demonstrates a novel and unique role for PFKFB3/iPFK2 in regulating adiposity and adipose tissue function, and thereby systemic insulin sensitivity. This role is manifested by the fact that disruption of PFKFB3/iPFK2 ameliorates HFD-induced adiposity, but exacerbates HFD-induced adipose tissue dysfunction, in particular, adipose tissue inflammatory response, which contributes to an increase in the severity of systemic insulin resistance. In contrast, adipocyte-specific PFKFB3 over-expression increased adiposity but suppressed overnutrition induced adipose tissue inflammatory response and improved insulin sensitivity. In addition, PFKFB3/iPFK2 involves in the anti-diabetic effect of PPAR γ activation. This role of PFKFB3/iPFK2 is evidenced by the fact that the intact PFKFB3/iPFK2 allows rosiglitazone to increase fat storage in the adipocytes/adipose tissue and to suppress adipocyte/adipose tissue inflammatory response. Mechanistically, the way by which PFKFB3/iPFK2 links two

adipose tissue-based mechanisms underlying the anti-diabetic effect of PPAR γ activation is attributable to the effect of PFKFB3/iPFK2 on regulating adipocyte lipogenesis and triglyceride synthesis as well as adipocyte fatty acid oxidation-related ROS production and inflammatory response (Fig. 46). These results indicate that selective activation of adipocyte PFKFB3/iPFK2 may be a viable approach to generating the beneficial effects of PPAR γ activation in the treatment of type 2 diabetes.

The mechanisms by which overnutrition induces intestine inflammation in relation to systemic insulin resistance and the actions of PPAR γ activation remain to be elucidated. PFKFB3/iPFK2 plays a critical role in protecting against diet-induced intestine inflammatory response. Notably, major intestine inflammatory biomarkers, including the mRNA levels of TLR4, TNF α and IL-6, as well as the phosphorylation of JNK1 and NF- κ B p65, in PFKFB3/iPFK2-disrupted mice were higher than their respective levels in wild-type controls under the condition of overnutrition, i.e., HFD feeding. Additionally, PFKFB3/iPFK2 appears to be needed for PPAR γ activation to fully suppress diet-induced intestine inflammatory response. Considering this, activation of intestinal PFKFB3/iPFK2 may be an approach to reversing overnutrition-associated intestine inflammatory response and to improving systemic insulin sensitivity.

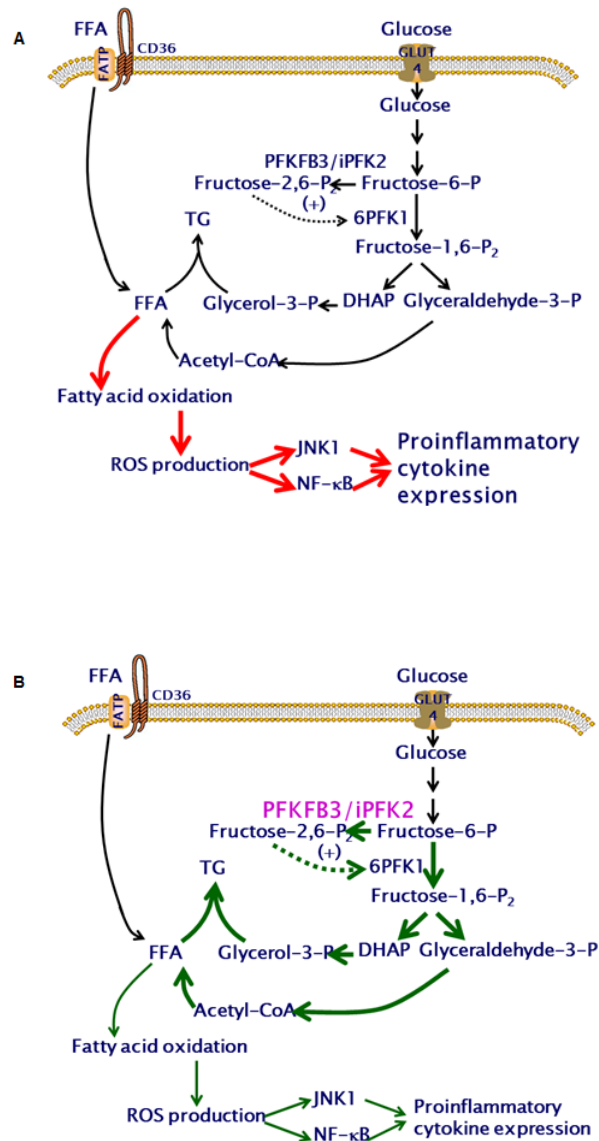


Figure 46. Involvement of PFKFB3/iPFK2 in the effects of PPAR γ activation in adipocytes. Under the condition of overnutrition (A), adipocytes exhibit an increase in inflammatory response, which is brought about at least in part by excessive fatty acid oxidation. Upon activation of PPAR γ (B), an increase in the expression of PFKFB3/iPFK2 enhances glycolysis to facilitate the synthesis of triglycerides (TG) via generating glycerol-3-phosphate and FFA (derived from acetyl-CoA following pyruvate oxidation). As a result, an increase in channeling FFA to triglyceride synthesis reduces fatty acid oxidation-associated production of ROS, thereby suppressing inflammatory signaling pathways through JNK1 and NF- κ B and decreasing the expression of proinflammatory cytokines. DHAP, dihydroxyacetone phosphate; GLUT4, glucose transporter 4; FATP, fatty acid transport protein.

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